VOLUME 30 FEBRUARY 1952 NUMBER 1

# *Canadian Journal of Zoology*

*Editor:* E. HORNE CRAIGIE

*Published by* THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

# CANADIAN JOURNAL OF ZOOLOGY

(*Formerly Section D, Canadian Journal of Research*)

The CANADIAN JOURNAL OF ZOOLOGY is published bimonthly by the National Research Council of Canada under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. Matters of general policy are the responsibility of a joint Editorial Board consisting of members of the National Research Council of Canada and the Royal Society of Canada.

The National Research Council of Canada publishes also: *Canadian Journal of Botany, Canadian Journal of Chemistry, Canadian Journal of Medical Sciences, Canadian Journal of Physics, Canadian Journal of Technology.*





**Manuscripts** should be addressed to:

Dr. Léo Marion,
Editor-in-Chief,
Canadian Journal of Zoology,
National Research Council,
Ottawa, Canada.

Each manuscript should be typewritten, double-spaced, and the original and one extra copy submitted (see **Notes to Contributors** inside of back cover).

**Subscriptions, renewals, and orders for back numbers** should be addressed to:

Administrative Services,
National Research Council,
Ottawa, Canada.

Subscription rate: $3.00 a year; single numbers: 50 cents. Special rates can be obtained for subscriptions to more than one of the Journals published by the National Research Council.


* *Deceased.*

VOLUME 30 1952

# *Canadian Journal of Zoology*

*Published by* THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Zoology

*Issued by* THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 30 FEBRUARY, 1952 NUMBER 1

# THE RELATIONSHIP OF THE SPRUCE BUDWORM (*CHORISTONEURA FUMIFERANA*, CLEM.) TO THE FLOWERING CONDITION OF BALSAM FIR (*ABIES BALSAMEA* (L.) MILL.)[1]

BY J. R. BLAIS[2]

## Abstract

Populations of the spruce budworm were studied on flowering and nonflowering balsam fir trees. Generally more eggs were found on the flowering trees. The flowering balsam fir trees were found to harbor higher populations in the early larval stages owing to the presence on these trees of staminate flowers and flower cups. The behavior of the larvae in relation to staminate flowers and flower cups was studied in both the field and the laboratory. Larvae that fed partially on pollen developed more rapidly than larvae that fed exclusively on foliage. Pollen as a food did not appear to have any direct effect on survival or fecundity. Other experiments showed that mortality was higher, development retarded, and fecundity reduced in insects forced to feed on old foliage in contrast with those fed on the current year's growth. Defoliation was more severe on flowering trees in the earlier stages of the infestation. However, as populations increased, wandering increased owing to competition for food. This resulted in an overflow of larvae from flowering to nonflowering trees.

## Introduction

During the past forty years the spruce fir stands of Eastern Canada have been subject to repeated attacks by the spruce budworm. These attacks have resulted in the loss of many millions of cords of wood.

Tothill (9) concluded that solid stands of balsam fir are mainly responsible for spruce budworm epidemics. Swaine and Craighead (8) first reported that spruce budworm epidemics seem to be associated with mature stands of balsam fir. Graham and Orr (3) reached the same conclusions.

Generally, the frequent and heavy production of flowers can be considered a characteristic of mature fir trees. The main object of the investigations reported here was to determine by field observations and experiments, the influence of flowering balsam fir on spruce budworm populations. Investigations were started in August, 1946, when four sample plots were established in the vicinity of the Wabigoon River in the Districts of Kenora and Sioux Lookout in northwestern Ontario. In 1947, five additional plots were established in the same general area (Fig. 1). Population studies on typical flowering and nonflowering balsam fir trees were carried out in 1947, 1948,

[1] *Manuscript received September 10, 1951.*

*Contribution No. 25, Division of Forest Biology, Science Service, Department of Agriculture, Ottawa, Canada. Based on a portion of a thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, University of Toronto, April, 1950.*

[2] *Research Officer, Forest Insect Laboratory, Sault Ste. Marie, Ont.*

[The December issue of this Journal (Can. J. Zool. 29 : 305-375. 1951) was issued January 8, 1952.]

and 1949. Experiments were carried out to determine the relations of the insect, at different stages of development, to the foliage and twigs of the two different categories of balsam fir.

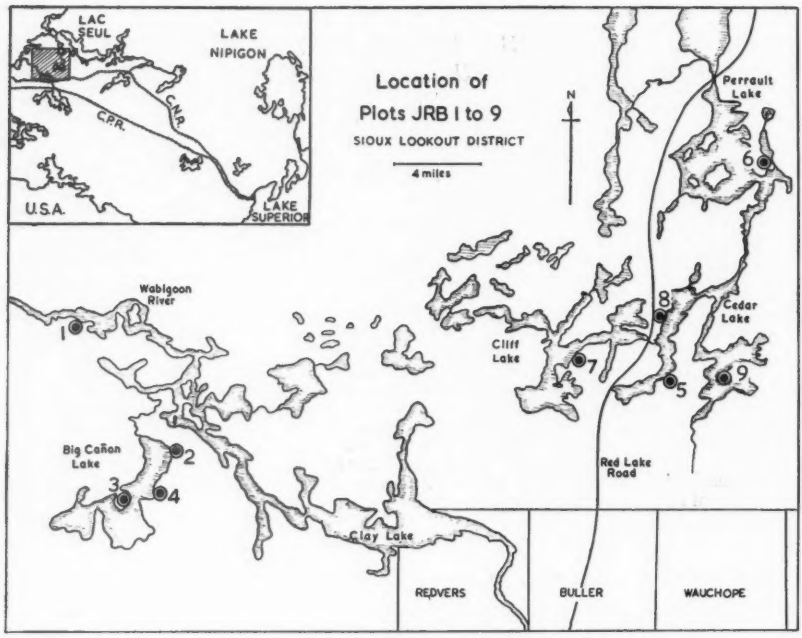



FIG. 1. Map showing the location of the study plots in northwestern Ontario.

## Material and Methods

The plots were established in stands varying in species composition, degree of infestation, age, and site quality to determine whether these factors affected the relations of the forest and the insect and to minimize the danger of generalizing on conditions that might be local.

Each stand in which plots were selected was thoroughly described. A stand table for trees from one inch upwards was prepared, based on detailed tally along a line or several lines one-half chain in width by 30 to 40 chains in length. Age and height of the important tree species were determined. Balsam fir trees were recorded as flowering, nonflowering, or intermediate. Some of the balsam fir trees were described as to total height, crown height, crown diameter, trunk diameter at breast height, and crown-class, and were tagged for yearly studies of defoliation and mortality. Soil conditions, ground cover, rate of growth, and other characteristics indicative of site quality were described. The past history of the stand was recorded as completely as possible.

In the analysis of the accumulated data, variations in age, site quality, and species composition of the forest were not found to affect the relations between the insect populations and the flowering condition of balsam fir. Therefore a detailed discussion of the variations in site, species composition, and other attributes of the forest is not included here.

In 1947 and 1948, the plots were visited in succession with the object of recording population density on the flowering and nonflowering trees in the larval, pupal, and egg stages of the budworm. Two or three trees of each category were studied on each visit to a plot. To avoid disturbance of the larvae and pupae during sampling, trees were lowered by a system of blocks and tackle (Fig. 2). From each lowered tree, nine 18-in. branch tips were

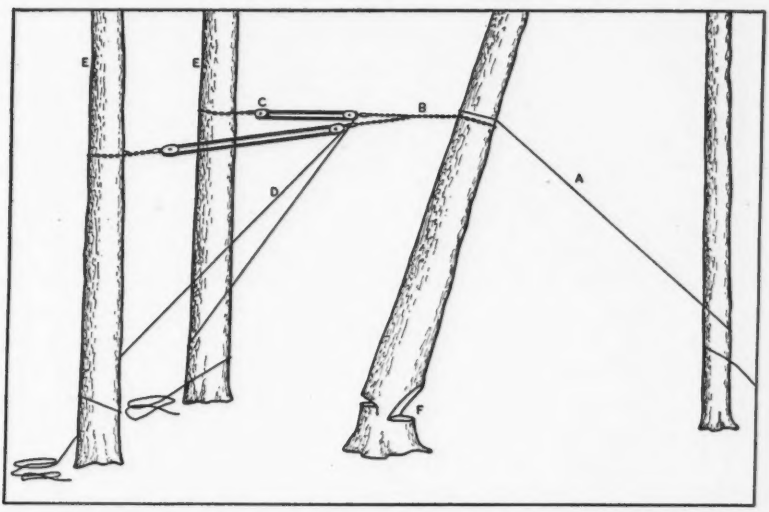



FIG. 2. Method used in lowering trees. The tree to be lowered is given a long, sloping undercut, *F*, and is supported by anchor trees, *E*, by a system of blocks and tackle, *B*, *C*, *D*. When everything is in readiness, the tree is pulled forward by pullrope, *A*, and the supporting tackle, *D*, released gradually.

taken at regular intervals from the tip to the bottom of the crown; each branch tip was placed separately in a numbered bag. The number of buds, number of buds attacked, number and size of flower clusters, length of shoots, and number and stage of the budworm were recorded for each branch. The position of the insects, whether in needles, buds, or flowers, was recorded. Details of procedure connected with other observations and experiments are described later.

## Description of Balsam Fir Trees, with Reference to Flowering

Typical flowering balsam fir trees differ from typical nonflowering trees in size, shape, type of foliage, shoot growth, number of buds for the same length

of branch, and flower production. Flowering trees on the average are larger in diameter, total height and crown length, and have a greater amount of "sun" foliage. The needles of sun foliage may be sharp or blunt, are frequently curved, and originate on all sides of the twig. The needles of "shade" foliage are long, flat, and blunt and are distributed in flat ranks along two sides of the twigs. The distribution of each type of foliage was recorded on one-eighth portions of the crown proceeding downward from the tip. The averages for these various measurements for the trees felled in 1947 and 1948 are shown in the following synopsis.

| No. of trees | Tree type | Av. d.b.h. | Av. total height | Av. crown length | Av. crown diameter | Foliage type 8ths of crown | | | Average age |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Sun | Int. | Shade | |
| 165 | F | 8.5 | 55 | 35 | 13 | 7 | 1 | — | 83 |
| 161 | NF | 6.5 | 39 | 29 | 12 | 5 | 2 | 1 | 70 |

Shoot length differs in flowering and nonflowering trees apparently owing to demands on food reserves made by developing flowers. In 1947, 2610 shoots from 29 flowering and 2520 shoots from 28 nonflowering trees in four plots had average lengths of 0.9 in. and 2.0 in. respectively. The relative shoot lengths of flowering and nonflowering trees vary from year to year, depending on the intensity of flower production. Nevertheless, the shoots of flowering trees are characteristically shorter than those of nonflowering trees (Figs. 3 and 4).

Usually flowering balsam fir trees are in the dominant and codominant crown classes whereas the nonflowering trees are in the intermediate and overtopped crown classes

Growth of the leader is relatively rapid in flowering trees, but radial growth of the crown is slow owing to poor shoot elongation. The crown has a "spire" top. The tops of nonflowering balsam fir trees are wide in relation to height. The branches of flowering trees have significantly greater numbers of buds per unit length than the branches of nonflowering trees. In 1947, the average number of buds per 18-in. branch tip on flowering trees, based on 988 branches, was $91 \pm 1.27$; on nonflowering trees based on 930 branches, the average was $56 \pm 0.91$. The number of buds on a branch is related to the total amount of foliage present. The buds were counted and the total length of foliated twigs was measured on 50 18-in. branch tips from each of two typical flowering and two typical nonflowering trees, yielding a correlation coefficient of 0.93 in the flowering trees and 0.69 in the nonflowering trees.

The staminate flower buds are formed in July, singly or in clusters, in rows along the twigs, usually in the upper half or two-thirds of the crown. The



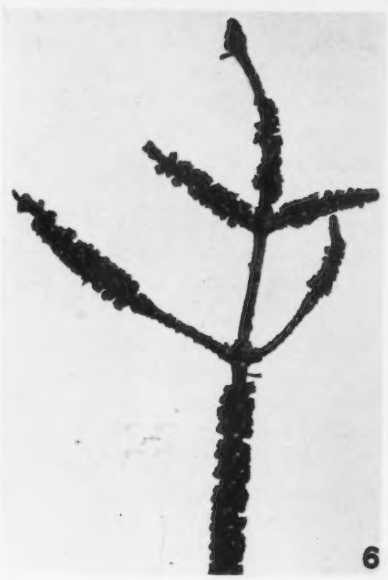



FIG. 3. Eighteen-inch branch tip from a nonflowering balsam fir. Fig. 4. Eighteen-inch branch tip from a flowering balsam fir. FIG. 5. Balsam fir branch showing staminate flower clusters as they appear in spring. FIG. 6. Balsam fir twig with needles removed to show the staminate flower scars.

following spring the flowers emerge from the scale covering, develop to about the size of a small pea, and are purple to mauve in color (Fig. 5). After two to three weeks, they expand and pollen is released over a period of three to four days. The spent flowers drop off leaving small cuplike scars (Fig. 6) which may persist on the branches for 25 years or longer. These scars roughen the surface of the twigs on flowering trees.

The ovulate cones are usually few and occur at the top of the crown. When the cone is ripe, the seeds and the scales are shed, but the central axis may persist as a thornlike stub for 30 years or longer.

Partial defoliation of the new foliage does not seem to affect the production of flowers severely. Some or all of the needles on a shoot may be destroyed, but providing the axis is not injured flower buds may appear along the shoot in late summer and bloom the following spring.

Typical flowering trees can be considered as physiologically mature. Physiological maturity is attained gradually, and is influenced not only by age but also by exposure to direct sunlight.

In the western regions of Ontario, balsam fir, if growing in full exposure to light, will attain maturity between the ages of 50 and 65 years, varying with site conditions. However, because of the great tolerance of this species to shade and to root competition, it can remain for years as a suppressed tree. A balsam fir tree suppressed for as long as 100 years may be only 1 or 2 in. in diameter at breast height, and only 10 or 15 ft. tall (6). Such a tree when released may assume the physiological characteristics of a young, vigorous tree with rapid shoot elongation and good diameter growth. It will normally proceed to maturity, and so to the regular production of flowers. The intermediate stage of flower production is relatively short; once a balsam fir has reached this status it becomes a typical flowering tree in a few years, often three or four.

In the western regions of Ontario, flowering balsam fir trees bear seed crops almost every year. The nonflowering trees may bear light crops at long intervals, as in "general seed years". These, in recent times, occurred in 1940, 1944, and 1948. Medium flowering occurred in 1947. Most of the typical flowering trees bore crops of variable size. Some of the nonflowering trees had no flowers, others had very light crops. In the general seed year, 1948, very heavy flowering occurred; typical flowering trees bore staminate flowers over most of their crowns, while the nonflowering trees bore lighter crops depending somewhat on the extent of crown exposure to direct sunlight. Because of the heavy production of flowers, up to 50% of the vegetative buds failed to develop on some of the flowering trees, and the shoots that did develop were short with short needles. On the nonflowering trees, few buds failed to develop, and shoot growth was much less severely affected. Owing to the complete destruction of the 1948 shoot growth, there were no flowers in 1949 in any of the plots.

## Relation of the Spruce Budworm to Balsam Fir

### Population Studies

*Larval and Pupal Populations*

In 1947 and 1948, populations on branches of equal length were greater on the flowering than on the nonflowering trees in the spring. As the season progressed populations dropped at a greater rate on flowering trees. In 1947, populations remained greater on flowering trees throughout the development of the insect; in 1948, at the end of the feeding stage, and through the pupal stage, nonflowering trees harbored higher populations (Figs. 7 and 8). Since

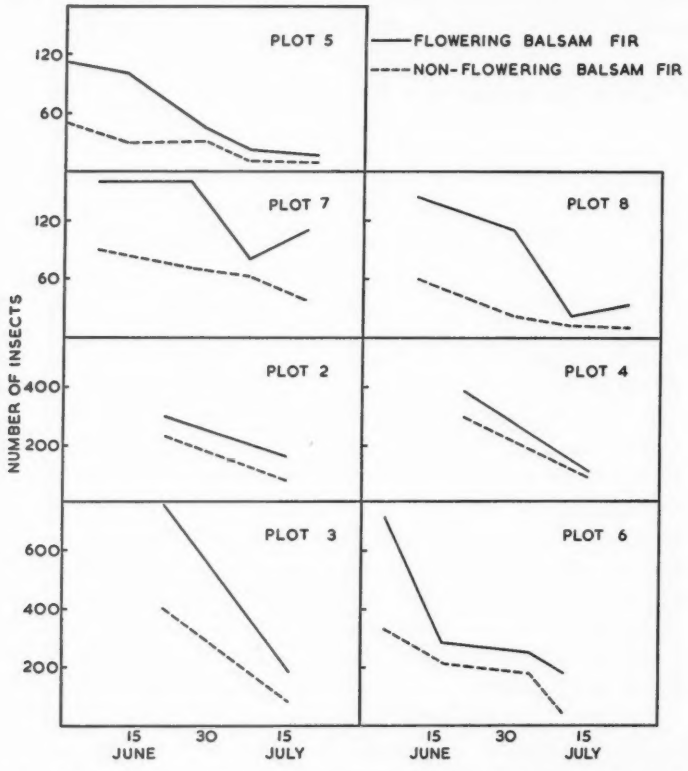



FIG. 7. Average number of insects on representative samples from flowering and nonflowering trees in 1947.

the flowering trees have larger crowns and more branches, the difference in population between flowering and nonflowering trees was even greater than would appear to be the case when comparisons are based solely on branches of equal length.

Analysis of the number of buds and number of insects in 1947 and 1948 revealed strong positive correlations in 25 of the 52 analyses. Although all but one of the remaining coefficients were positive, they were not statistically

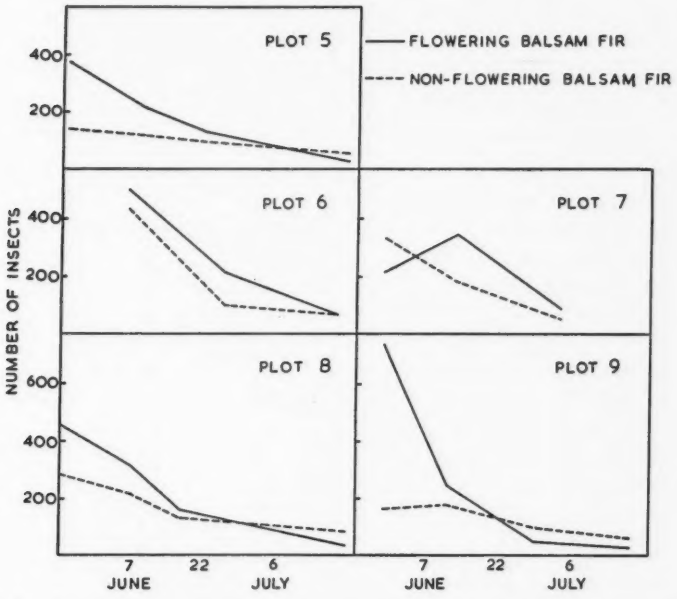



FIG. 8. Average number of insects on representative samples from flowering and non-flowering trees in 1948.

significant. In 1947, there was a significant correlation between insects and buds in the pupal, as well as in the larval stage (Table I). In 1948, the number of pupae was not significantly correlated with the number of buds.

Since the flowering trees had significantly more buds per unit length of branch than the nonflowering trees, it is logical to question whether the increased number of buds could have accounted for the increased number of insects on the flowering trees. Undoubtedly, the greater number of buds on flowering trees contributed to higher budworm populations, but when an analysis of covariance (7) was applied to the data, it was apparent that some factor other than the number of buds was responsible for higher populations on the flowering trees during the feeding stages of the insect. Table I shows the regression equations for the 1947 data. The data for the larval and pupal stages in 1948, and for the egg stage in 1947, 1948, and 1949, were submitted to the same type of analysis.

The tests of significance of the difference between the adjusted mean number of insects to mean number of buds on flowering and nonflowering trees for 1947 show that there was a significant difference in the number of insects

TABLE I

RELATION OF THE NUMBER OF INSECTS TO THE NUMBER OF BUDS (OR NEW SHOOTS) ON FLOWERING AND NONFLOWERING BALSAM FIR TREES IN THE STUDY PLOTS IN 1947

(Nine 18-in. branches constituted the sample from each of two or three trees of each category at each time of sampling. Collections from June 1–July 6 consisted of larvae, from July 12–16 of pupae)

| Date | Plot No. | Tree category | Mean no. of buds or shoots per branch ($x$) | Mean no. of insects per branch ($y$) | $r_{yx}$ | Regression equation estimation of $y$ |
|---|---|---|---|---|---|---|
| June 1 | 5 | F | 97.4 | 11.8 | .03 | $10.9 + .01x$ } (3) |
| | | NF | 98.6 | 5.6 | .71(1) | $-2.2 + .08x$ } |
| June 4 | 6 | F | 122.1 | 79.4 | .38 | $30.6 + .40x$ } (3) |
| | | NF | 80.9 | 36.7 | .48(2) | $11.6 + .31x$ } |
| June 8 | 7 | F | 88.6 | 18.8 | .28 | $12.6 + .07x$ } (3) |
| | | NF | 94.6 | 11.1 | .51(1) | $-0.2 + .12x$ } |
| June 12 | 8 | F | 117.0 | 16.2 | .59(1) | $-2.5 + .16x$ } (3) |
| | | NF | 94.9 | 6.8 | .40 | $1.1 + .06x$ } |
| June 14 | 5 | F | 115.4 | 9.9 | .61(1) | $-2.8 + .11x$ |
| | | NF | 54.0 | 3.8 | .41 | $-2.1 + .11x$ |
| June 21 | 2 | F | 78.7 | 35.0 | .58(1) | $5.9 + .37x$ } (3) |
| | | NF | 94.7 | 29.0 | .49(2) | $3.4 + .27x$ } |
| June 26 | 7 | F | 95.1 | 19.5 | .36 | $11.0 + .09x$ } (3) |
| | | NF | 67.1 | 8.5 | .29 | $4.1 + .07x$ } |
| June 30 | 8 | F | 101.2 | 9.7 | .65(1) | $-0.4 + .10x$ } (3) |
| | | NF | 66.1 | 2.5 | .53(1) | $-0.8 + .05x$ } |
| July 4 | 6 | F | 91.7 | 28.5 | .68(1) | $1.0 + .30x$ |
| | | NF | 63.0 | 21.3 | .62(1) | $-1.4 + .36x$ |
| July 6 | 7 | F | 75.3 | 8.0 | .30 | $3.5 + .06x$ |
| | | NF | 60.0 | 7.7 | .10 | $6.5 + .02x$ |
| July 12 | 6 | F | 118.6 | 20.9 | .72(1) | $-8.7 + .25x$ |
| | | NF | 40.2 | 4.1 | .33 | $0.9 + .08x$ |
| July 16 | 2 | F | 88.9 | 17.1 | .56(1) | $-1.6 + .21x$ |
| | | NF | 53.5 | 8.2 | .56(1) | $2.9 + .10x$ |

NOTE: (1) *Significant at .01.*

(2) *Significant between .01 and .05.*

(3) *The test between the adjusted means shows significantly more insects on the flowering balsam fir trees.*

on the two types of trees up to the end of June. There were significantly more insects on flowering trees than on nonflowering trees, from the time of establishment of second-instar larvae to the time when the majority of the larvae were in the sixth instar. At this time, the extensive intertree wanderings began to affect the distribution of populations, especially in areas of heavy

infestation. Although there were still more insects on flowering trees, when compared on a per bud basis the difference was no longer significant.

In 1948, the pattern of population distribution was similar to that of the previous year. Some differences occurred, however, as a result of differences in tree growth and insect populations as described later. That year, in all of the samples except one from Plot 5 and one from Plot 6, budworm larvae were significantly more abundant on a per bud basis on the flowering trees from May 11 until June 9, when the majority of the insects were in the fifth instar. Later the differences in population were either not significant or were reversed. In two of the plots, populations were significantly greater on the nonflowering trees at the time of the sixth instar and the pupal stage.

*Egg Populations*

The flowering trees harbored higher populations of eggs, per unit length of branch, than did the nonflowering trees, in plots studied in 1946, 1947, 1948, 1949, and 1950, with one exception (Fig. 9). Correlation analyses of the number of buds and egg clusters for 1947 and 1948 revealed strong positive correlation in 11 of the 38 analyses. All but three of the remaining 27 coefficients were positive, but were not significant.

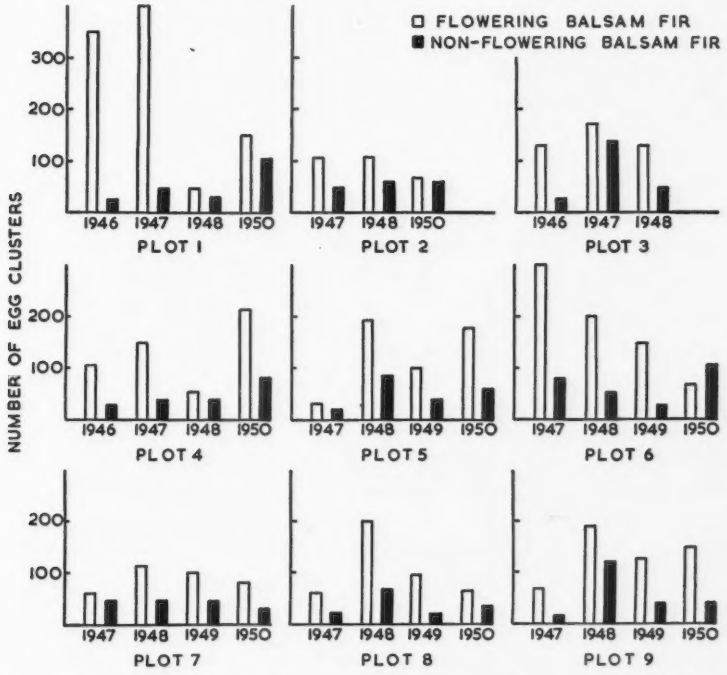



FIG. 9. Average number of egg clusters on representative samples from flowering and nonflowering trees, 1946 to 1950.

Although there is some evidence that the number of egg clusters may be correlated with the number of buds, this does not provide an adequate explanation of the larger number of eggs found on flowering trees. Covariance analyses showed significantly more egg clusters on flowering trees than would be expected solely on the basis of the number of buds. Evidently, in areas of heavy infestation ovipositing moths were attracted to flowering trees by some factor other than the amount of available foliage as reflected by the number of buds.

Unfortunately, the data taken in 1946 were not amenable to analysis of covariance. However, the number of egg clusters per bud indicates that on a per bud basis the flowering trees harbored higher populations than the nonflowering trees in the plots studied. There were approximately five times as many egg clusters per bud on the flowering as on the nonflowering trees in Plot 1 and approximately twice as many on the flowering as on the nonflowering trees in Plots 2 and 3.

In 1947, in those plots where the infestation was heavy (Plots 1, 2, 3, 4, and 6) the flowering trees harbored a significantly higher egg population on a per bud basis than did the nonflowering trees, as shown by analysis of covariance. There was no difference in number of egg clusters on a per bud basis between the two categories of balsam trees in those plots where the infestation was light to medium (Nos. 5, 7, 8, and 9).

In 1948 all the plots were heavily infested and the ovipositing moths showed a preference for the flowering trees. A significantly higher egg population, on a per bud basis, occurred on the flowering trees in all the plots but Plot 1. Defoliation in Plot 1 had been so severe for the previous three years that some trees were almost completely defoliated, and this plot was no longer comparable with the others.

By 1949, because of past defoliation, many adventitious buds had been produced. This distorted the normal distribution pattern of buds on the sample branches. That year, each branch was measured so that the total length of the foliated twigs was obtained but the buds were not counted. In all the plots, a stronger and much more constant correlation existed between the number of egg clusters and the number of inches of foliated twigs than had previously occurred between egg clusters and buds. The correlation was highly significant in all but one case. In only one of the six plots studied in 1949 did flowering trees harbor significantly more eggs per inch of foliated twig than nonflowering trees.

The following synopsis gives the mean number of eggs per cluster on flowering and nonflowering trees for 1947 and 1948. The differences in cluster size were not significant.

| Year | Category of tree | Number of egg clusters | Average number of eggs per cluster |
|---|---|---|---|
| 1947 | F | 100 | 19.1 ± 1.1 |
| | NF | 85 | 21.8 ± 1.2 |
| 1948 | F | 175 | 18.8 ± 0.8 |
| | NF | 200 | 16.9 ± 0.8 |

## Insect Behavior

### *Tree Selection by Ovipositing Moths*

The egg populations on the two types of trees in 1946, 1947, 1948, 1949, and 1950, have been described in the preceding section. Although the relative population densities varied somewhat, the ovipositing moths generally showed a preference for the flowering trees, especially in areas of heavy infestation.

This preference for flowering trees during oviposition was most striking in 1948 when eight of the nine plots showed more eggs on a per bud basis on the flowering than on the nonflowering trees. Considering that the pupal population in 1948 was greater on the nonflowering trees, the fact that more eggs were found on flowering trees is especially significant in view of the tendency of female moths not to fly until after some oviposition has occurred (11). The number of empty pupal skins and the number of egg clusters were counted on each of three flowering and each of three nonflowering trees in each of four plots in 1948. The ratios between the number of egg clusters and the number of pupal cases were greater for flowering trees as shown in the following synopsis and indicate that the moths preferred these trees for oviposition.

| Plot No. | Tree type | No. of egg clusters per pupal case |
|---|---|---|
| 5 | F | 5.7 |
| | NF | 1.6 |
| 6 | F | 4.0 |
| | NF | 1.2 |
| 8 | F | 6.5 |
| | NF | 0.8 |
| 9 | F | 8.5 |
| | NF | 2.3 |

In 1949, an attempt was made to determine experimentally why the ovipositing moths showed this preference. In one experiment, two 6-in. twigs, one with flower scars from the flowering type, and the other from the nonflowering type of balsam fir, were placed in lantern globes with a mated female moth. A total of 75 globes was set up in this manner. When oviposition was complete, the number of egg clusters and eggs were counted on each twig. A comparison of the mean number of eggs laid on twigs from the flowering trees ($52.5 \pm 6.9$) and the mean number of eggs laid on twigs from the nonflowering trees ($74.4 \pm 6.4$), shows a significant difference in favor of the latter. This would seem to eliminate the possibility that ovipositing moths are attracted by flower scars. The greater number of eggs on the twigs from nonflowering balsam trees might be explained by the fact that these twigs had not been subjected to as heavy defoliation as the twigs from the flowering trees.

In another experiment, one 12-in. branch of each type was placed in a screen cage, 1 × 1 × 2 ft. The cut ends of the branches were immersed in water. A total of 25 male and 25 female pupae was placed on a small tray in the middle of the cage between the two branches. Four cages were set up in a canvas tent so as to avoid any point source of light that might act on the light tropism of the moths and affect the direction of flight. After oviposition was complete the egg clusters were counted and the total length of foliated twigs for each branch was measured. There was an average of 0.56 egg clusters per inch of foliated twigs for flowering trees and 0.51 egg clusters per inch of foliated twigs in the case of nonflowering trees. Results show a random distribution of egg clusters, when compared on the basis of equal lengths of foliated twigs from each type of branch, and suggest that the ovipositing moths do not seem to be attracted to flowering balsam trees because of the type of foliage or the presence of flower scars.

A third experiment was carried out with 5-ft. sections from the top of the crowns of two typical flowering and two typical nonflowering trees. These were placed upright, one in each corner of a large screen cage, 11 × 11 × 6 ft. So as not to have two similar categories on the same side of the cage, the tree sections were placed alternately. A tray with 1000 budworm pupae was suspended from the center of the cage. When oviposition was complete, egg clusters were counted from the top, middle, and bottom whorl of branches from each tree section, and the total length of foliated twigs was measured. On all sections there was a definite gradation in the number of egg clusters laid in the top, middle, and lower branches, more eggs occurring in the upper sections. Evidently the distribution of the egg clusters on the different tree sections was determined to a much greater extent by the orientation of these tree sections within the cage than by their flowering characteristics. Many more eggs were deposited on the two sections in the southeast and the southwest side of the cage than on the two sections in the northeast and northwest side.

More eggs are generally found on flowering trees, possibly because they are taller and grow more in the open than the nonflowering trees. These features, rather than the physical characteristics of the branches or the foliage, would appear to be the important factors.

*Selection of Hibernation Sites*

On emerging from the eggs in midsummer the larvae crawl and drop on silk threads in search of proper niches for the construction of hibernacula. In the course of this wandering many larvae are carried away by air currents and many are lost (4).

Bess (2) mentions that staminate flower cups are especially favored by the larvae as niches in which to form their hybernacula. Experiments were performed to measure the ease with which first-instar larvae established hibernacula on twigs with and without staminate flower scars. In one experiment, involving 24 replications, 10 newly emerged larvae were placed on twigs of the two types. Each twig was 4 in. long and secured in an upright position.

The position and behavior of each larva were recorded at 15-min. intervals for a period of two and one-half hours. The following synopsis gives an account of the larvae on each type of twig at the end of the period.

| Type of twig | Established hibernacula | Still wandering | Left twig | Unaccounted for |
|---|---|---|---|---|
| F | 71% | 6% | 12% | 11% |
| NF | 14% | 19% | 59% | 9% |

Sixty per cent of the insects placed on twigs with flower scars had spun hibernacula in the flower scars and 11% between them. Most of these larvae established themselves almost immediately on being placed on the twigs. None established hibernacula in the axils of the needles. The larvae placed on twigs without flower scars established themselves in the axils of needles after much wandering. Those that abandoned the twigs did so by crawling or spinning off.

In a second experiment, an egg cluster was placed an equal distance from the base of two 1½ in. twigs, one of each type, cemented upright in the bottom of an 8-oz. jar. Eighteen jars were set up in this manner. The jars were then sealed and left in the dark to avoid the effect of any light reaction on the direction of wandering of the emerging larvae. An account of the 379 larvae several days after emergence from the egg clusters follows.

| Established in flower scars | Established on NF twigs | Established on wall of jar | Still wandering | Dead | Unaccounted for |
|---|---|---|---|---|---|
| 64% | 1% | 12% | 10% | 2% | 11% |

Most of the hibernacula on the walls of the jar occurred at the edge of the lid.

The preference of hibernating larvae for branches with flower scars was shown by an experiment with balsam fir foliage obtained from the field. In early May, 1948, nine 18-in. branches were taken from each of three flowering and three nonflowering trees. The buds were counted, and the branches placed singly in waxed cartons with a test tube stuck through the lid to collect the emerging second-instar larvae. The essential data follow:

| Tree type | No. of buds | No. of emerged larvae | No. of larvae per 100 buds |
|---|---|---|---|
| F | 2992 | 657 | 22 |
| NF | 2735 | 112 | 4 |

Significantly more larvae emerged from flowering than from nonflowering trees, on a per bud basis. These samples had been selected in Plot 8 where extensive egg counts in 1947 showed that there was no significant difference, on a per bud basis, between the number of eggs on flowering and nonflowering trees. Conditions between the time of hatching in 1947 and emergence from the hibernacula in 1948 must therefore have favored the concentration or the survival of populations on the flowering trees.

A similar experiment was carried out with branches collected in Plot 8 in the fall of 1948, and held in cold storage until April, 1949. As before, nine 18-in. branches were taken from each of three flowering and three nonflowering trees. Each branch was placed individually in a container at room temperature; a little water was sprinkled on the branches every second day. Emergence began in a week and extended over a period of 10 days. Approximately four times as many larvae emerged per bud from the flowering trees as from the nonflowering trees (Table II).

TABLE II

EMERGENCE OF SECOND-INSTAR LARVAE FROM NINE 18-IN. BRANCHES FROM EACH OF THREE FLOWERING AND THREE NONFLOWERING BALSAM TREES IN APRIL, 1949

| Tree type | Tree No. | Number of buds | Number of larvae emerged from hibernacula | Number of larvae per 100 buds |
|---|---|---|---|---|
| F | 1 | 922 | 1236 | 134 |
| | 2 | 876 | 865 | 99 |
| | 3 | 1108 | 946 | 85 |
| Totals | | 2906 | 3047 | 105 |
| NF | 4 | 444 | 74 | 17 |
| | 5 | 401 | 119 | 30 |
| | 6 | 828 | 248 | 30 |
| Totals | | 1673 | 441 | 26 |

Other branches were collected in Plot 8 in the fall of 1948 and held in cold storage until April, 1949. Branches from flowering trees were cut up into sections with flower scars, and others without flower scars; in each case the total length of the sections was 535 in. Branches from nonflowering trees were treated similarly and the total length of twig sections was 330 in. in each case. Table III shows that many more larvae had hibernated successfully in those sections of the branches with flower scars.

It is concluded that the flowering balsam fir trees present conditions which are more favorable to the overwintering larvae and thereby contribute to a higher survival of the insect at that stage. This conclusion is at variance with that of Jaynes and Speers (5) who suggest that little preference is shown for staminate bracts as a place of hibernation. Their conclusion, however, is based on a very limited experiment.

TABLE III

EMERGENCE OF SECOND-INSTAR LARVAE FROM SECTIONS OF BRANCHES* WITH FLOWER SCARS AND WITHOUT FLOWER SCARS FROM TYPICAL FLOWERING AND TYPICAL NONFLOWERING BALSAM FIR TREES IN APRIL, 1949

| Tree type | Twig sections with flower scars | | | Twig sections without flower scars | | |
|---|---|---|---|---|---|---|
| | Inches of twigs sampled | No. of larvae emerged | No. of** larvae | Inches of twigs sampled | No. of larvae emerged | No. of** larvae |
| F | 535 | 319 | 60 | 535 | 24 | 4 |
| NF | 330 | 601 | 182 | 330 | 49 | 15 |
| Total | 865 | 920 | 106 | 865 | 73 | 8 |

* *Because of the relative scarcity of flower scars on branches from nonflowering trees, these sections had to be obtained from a larger number of branches than in the case of those obtained from flowering trees.*

** *Number of larvae expressed as the number per 100 in. of foliated twigs.*

### *Selection of Feeding Sites*

Extensive wandering and dispersal again take place in the spring when, on emerging from hibernation, the larvae wander about in search of food. The only succulent food available at this time is the staminate flowers. These are swollen for some days prior to budworm emergence, whereas the vegetative buds do not burst for one to two weeks following emergence.

Until recently it was generally believed that in the spring the second-instar larvae, on emerging from their hibernacula, would bore directly into buds (3), or mine the needles of old foliage (1). The present study indicates that where staminate flowers are available, large numbers of the larvae never go through the needle-mining phase.

Staminate flowers at Cedar Lake were swollen by May 13, 1947. The first signs of budworm activity were noted on May 23, when emerging second-instar larvae were mining balsam needles and swollen staminate flowers. It was not until June 6, that vegetative buds were starting to swell and burst.

In 1948, budworm emergence began on the morning of May 12. By midday large numbers were crawling on branches and spinning webs. Many larvae were established in staminate flowers by midafternoon. When branches bearing flowers and others with no flowers were examined, 218 larvae were found in flowers and only three larvae in needles. This preference for staminate flowers persisted up to the time of shedding of pollen some three weeks later.

Table IV shows the distribution of young budworm larvae on flowering and nonflowering trees in 1947 and 1948. Flower clusters were classified as small, medium, or large, depending on whether they extended for less than one-half inch, between one-half inch and one inch, or over one inch, along the

twigs. The number of individual flowers in clusters of the different classes were counted, with the following results:

| Size of flower cluster | No. examined | Mean no. of flower buds | Relative no. of flower buds |
|---|---|---|---|
| Small | 139 | 8.09 ± .51 | 1.0 |
| Medium | 178 | 18.93 ± 1.25 | 2.4 |
| Large | 138 | 28.50 ± .67 | 3.6 |

The relative index of flower production in Table IV was obtained by multiplying the number of small, medium, and large flower clusters by 1, 2.4, and 3.6 respectively.

There is a strong tendency for the majority of the young larvae to occur in the flowers, especially where the flowers are abundant.

The relative ease with which emerging second-instar larvae establish themselves in staminate flowers, and in needles, was observed in the spring of 1948. Three-inch twigs were placed singly in inverted No. 3 vials, 20 of which contained twigs with flowers, and an equal number contained twigs bearing 1947 foliage but no flowers. The base of each twig was kept in water. Seven newly emerged larvae were placed in each vial. In the vials with flowering twigs, the larvae almost immediately began to spin webbing and establish themselves, while in those with 1947 needles only, far more wandering was observed. An account of the larvae on May 18, four days after setting up the experiment, is shown below. The second-instar larvae established themselves more readily and more successfully in flowers.

| Type of food | Original population | Established in flowers | Established in needles | Roaming | Dead |
|---|---|---|---|---|---|
| Flowers | 140 | 132 | 2 | 2 | 4 |
| Foliage | 140 | — | 106 | 19 | 15 |

In 1949, no flowers were produced on balsam fir in the study area. This presented an opportunity for the study of the distribution of populations during the needle-mining phase. Populations on advance balsam fir growth were studied in four different areas with varying degrees of defoliation (Table V). Second-instar larvae mined the needles of the most recent year of growth. In the Perrault Lake area, all the 1947 and 1948 foliage had been destroyed; however, an occasional adventitious shoot had been produced in 1948 after feeding by the budworm was over. Nearly every needle on these shoots was mined in 1949 in this area of relatively light infestation. The high proportion of needles mined in the few adventitious shoots indicates that the larvae wander considerably in search of the most recent growth.

TABLE IV

EARLY DISTRIBUTION OF LARVAE ON FLOWERING AND NONFLOWERING BALSAM FIR TREES IN 1947 AND 1948

| Year | Date | Plot No. | Tree type | No. of trees | Relative index of flower production* (for nine 18-in. branches) | Av. no. larvae (for nine 18-in. branches) | Per cent population in | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Fowers | Needles | Buds |
| 1947 | May 28 | 8 | F | 1 | 592 | 136 | 85 | 15 | — |
| | | | NF | 1 | 0 | 18 | — | 100 | |
| | June 1 | 5 | F | 3 | 455 | 106 | 44 | 56 | — |
| | | | NF | 3 | 28 | 50 | 16 | 84 | — |
| | June 4 | 6 | F | 3 | 272 | 684 | 37 | 60 | 3 |
| | | | NF | 3 | 16 | 473 | 4 | 91 | 5 |
| | June 8 | 7 | F | 2 | 847 | 170 | 84 | 16 | — |
| | | | NF | 2 | 16 | 100 | 3 | 94 | 3 |
| | June 12 | 8 | F | 2 | 229 | 146 | 56 | 12 | 32 |
| | | | NF | 2 | 92 | 61 | 23 | 25 | 52 |
| | June 14 | 5 | F | 4 | 228 | 88 | 40 | 5 | 55 |
| | | | NF | 3 | 8 | 29 | 5 | 8 | 87 |
| 1948 | May 20 | 8 | F | 3 | 1182 | 461 | 95 | 5 | — |
| | | | NF | 3 | 484 | 280 | 52 | 46 | 2 |
| | May 24 | 5 | F | 2 | 2005 | 462 | 99 | 1 | — |
| | | | NF | 2 | 122 | 233 | 51 | 32 | 17 |
| | May 28 | 9 | F | 2 | 1633 | 763 | 92 | — | 8 |
| | | | NF | 2 | 358 | 169 | 68 | 11 | 21 |
| | May 31 | 7 | F | 2 | 1335 | 461 | 70 | — | 30 |
| | | | NF | 2 | 0 | 280 | — | — | 100 |

* *For explanation, see text.*

TABLE V

DISTRIBUTION OF SECOND-INSTAR LARVAE IN THE SPRING OF 1949 IN NEEDLES PRODUCED IN DIFFERENT YEARS, AS INFLUENCED BY SEVERITY OF DEFOLIATION IN PREVIOUS YEARS

| Locality and degree of previous defoliation | Number of larvae observed | Per cent of population in needles produced in | | | | |
|---|---|---|---|---|---|---|
| | | 1948 | 1947 | 1946 | 1945 | 1944 |
| Cliff Lake<br>No previous defoliation | 185 | 100 | 0 | 0 | 0 | 0 |
| Plot 8<br>Defol. of 1948 growth (60–70%)<br>Defol. of 1947 growth (10%) | 434 | 83.2 | 16.6 | 0.2 | 0 | 0 |
| Jackfish Lake<br>Defol. of 1948 growth (100%)<br>Defol. of 1947 growth (25%) | 250 | — | 85.6 | 14.0 | 0.4 | 0 |
| Perrault Lake<br>Defol. of 1948 growth (100%)<br>Defol. of 1947 growth (100%)<br>Defol. of 1946 growth (10%) | 180 | — | — | 85.0 | 14.4 | 0.6 |

The apparent preference of second-instar larvae for needles of the most recent growth may be of importance in bringing about the decline of populations. In 1949, there was a definite subsidence of the infestation where populations had been most active for the longest period of time. This was reflected in the defoliation of the current year's growth in Plots 1, 3, 4, and 6 (Table VII). In these plots all the 1947 and 1948 growth had been destroyed on both categories of trees. In addition, no flowers were present in 1949 so that many larvae emerging in the spring probably failed to establish themselves for lack of proper food. In 1950, some staminate flowers as well as needles of the previous year's growth were available to the larvae in these same areas and populations once again built up to the point where almost all the current year's growth was destroyed.

*Feeding Habits of Third- to Sixth-instar Larvae*

The preference of the larvae for succulent foliage persists throughout the feeding stage and determines, to a large degree, insect movements and distribution of populations. Before accepting old foliage, spruce budworm larvae do a considerable amount of wandering or spinning down in search of new growth.

When the insects are not sufficiently numerous to cause competition for food, wandering is relatively unimportant. When competition is severe, as

during epidemics, wandering is very common. In 1947 and 1948, the behavior of budworm larvae was observed in the field in different plots under different conditions. Both the field observations and the systematic population counts showed that, in all cases during the feeding stage, budworm wanderings and the distribution of populations were closely associated with the availability and distribution of new growth.

As already mentioned, 1947 was a year of medium flower production in balsam fir in northwestern Ontario and shoot growth was good. That year, the more intensive studies were carried out in plots where budworm populations were not sufficiently heavy to cause complete defoliation of the new growth (see Plots 5, 7, 8, and 9, Table VII). In the early part of the feeding period, up to and including the fourth instar, populations were concentrated at about the middle third of the tree crowns, where male flowers were produced. After the shedding of pollen and the partial destruction of the shorter shoots in that part of the crown, there was a very noticeable intratree movement of the larvae to the upper third and the lower third of the crown where new growth was available. As this supply of foliage was reduced, intertree wandering of the fifth- and sixth-instar larvae was commonly observed. This accounted for the pattern of population distribution in 1947 (Fig. 7) already described; namely, that in all the developmental stages the budworm was more abundant on flowering than on nonflowering balsam trees, and that the populations on a per bud basis remained significantly higher on flowering trees up to the time of the sixth instar. Thereafter, owing to intertree wanderings, there was no longer any significant difference between populations on the flowering and nonflowering trees.

Because 1948 was a heavy seed year for balsam fir, shoot growth was severely reduced. Also, populations were much higher in those plots where, in 1947, the infestation had been light to medium. The reduction in available food and the increase in population resulted in acute competition and subsequent larval wanderings. Early in the season, the populations were found in the part of the crowns bearing staminate flowers. This comprised almost the whole of the crown of typical flowering trees, and varying proportions of the crown of the nonflowering trees, depending on their exposure to direct sunlight. After the shedding of pollen, very little new growth was available on most flowering trees and intertree wandering began at the time of the fourth instar. The nonflowering balsam fir trees, white spruce, black spruce and advance growth of these three species, had good shoot growth and were invaded by large numbers of budworm larvae in the late fourth and subsequent instars. The time when intertree wandering occurred varied with the different plots (Fig. 8). It occurred earliest in Plot 7 where the heavy production of flowers and poor growth conditions resulted in an extremely meager shoot growth on flowering trees. Larvae left these trees in large numbers at the time of the fourth instar. In the other plots the nonflowering trees did not harbor higher populations than the flowering trees until the sixth instar.

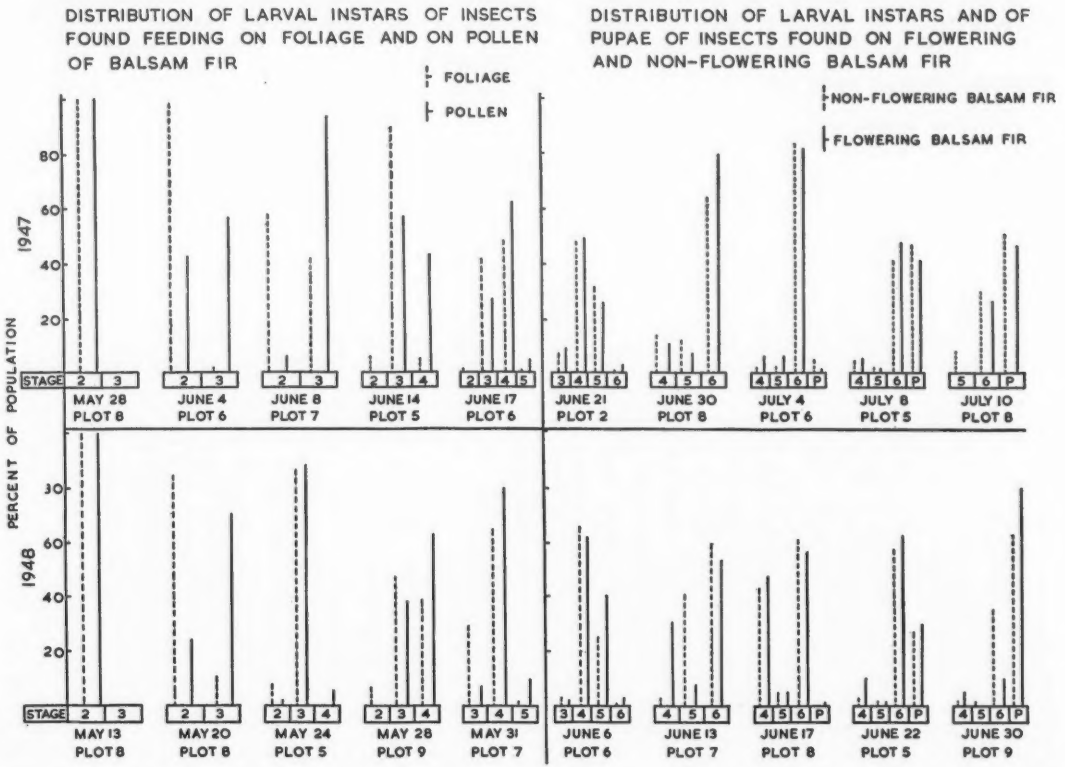



FIG. 10. Distribution of instars of the spruce budworm on flowering and nonflowering balsam fir trees, 1947 and 1948.

## Development, Survival, and Fecundity in Relation to Food

The larvae feeding on pollen and those feeding on foliage showed a difference in rate of development up to and including the time of the fourth instar. Fig. 10 shows the development of larvae in the field in 1947 and 1948. After June 17, 1947, and May 31, 1948, the pollen was shed, the spent flowers were abandoned for terminal buds, and wandering within the tree and from tree to tree increased and continued to the time of pupation. As a result, the larvae that were initiated on pollen became intermixed with those that started feeding in needle mines. The development of larvae found on flowering and nonflowering balsam trees was similar after June 21, 1947, and after June 6, 1948.

Wellington (10) has shown that male flowers warm up more rapidly and retain heat longer than do foliage buds or needles when exposed to solar radiation. This would seem to explain why, in the field, larvae feeding in staminate flowers develop faster than those feeding on foliage.

In order to determine whether the advantage gained by those insects which were initiated on pollen persisted, 250 larvae were collected from staminate flower clusters and 250 from foliage prior to the shedding of pollen in 1948. They were reared to adults in 8 oz. screw-top jars, 10 per jar, and were examined every third day when the food was changed. All the larvae were fed balsam shoots of the current year's growth. Fig. 11 shows the development of the reared insects from June 3 to the time of pupation. Daily records of

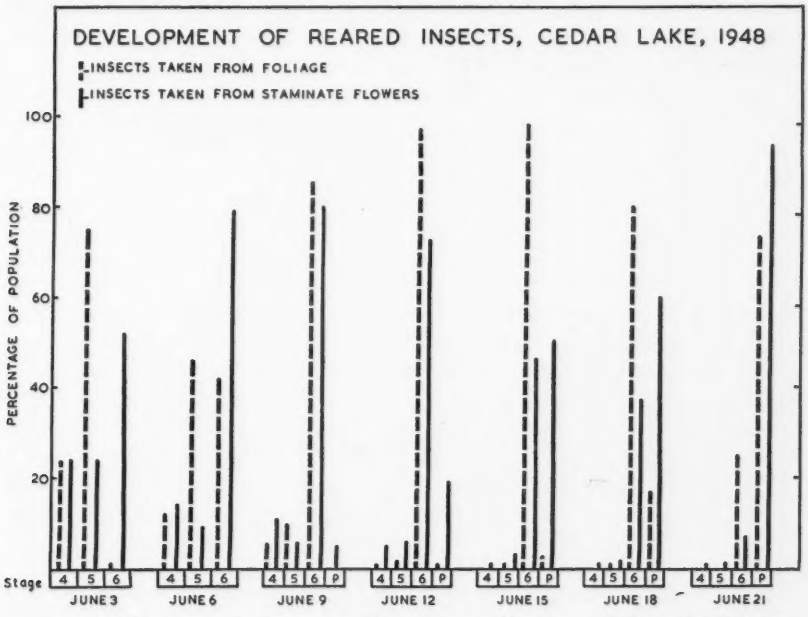



FIG. 11. Distribution by instars, on different dates, of budworm samples collected from foliage and from staminate flowers and reared in the laboratory on balsam fir foliage until maturity.

emergence were maintained. Male moths of insects originally from staminate flowers emerged three to four days ahead of those originally from foliage. The female moths of insects from the staminate flowers emerged approximately two days ahead of those from foliage. Jaynes and Speers (5) arrive at the same conclusion, that larvae feeding on pollen obtained a slight advantage and maintained this lead over those feeding only on the vegetative buds.

Because budworm larvae are occasionally forced to feed on foliage other than the current year's growth and because this affects wandering and eventual distribution of populations, special studies were carried out in 1949 to measure the effect of old foliage on the rate of development, survival and fecundity of the insect. Four hundred larvae were collected in each of the third, fourth, fifth, and sixth instars. From each lot, 200 larvae were reared on 1948 foliage and 200 on 1949 foliage, in lots of 10 in 8-oz. jars. In addition, 200 larvae obtained from the field in the fifth instar were reared on 1947 foliage. All foliage used in the experiment was obtained from the same tree. The food was changed every third day, and the number of insects and the developmental stage of each individual was recorded. Because the rate of development of insects is affected by parasitization, parasitized insects were deleted from the calculations.

The development of the insects reared on old foliage was greatly retarded and the difference in development increased with the time the larvae had been subjected to a diet of old foliage, and also with the age of the foliage. Mortality was so high among larvae collected in the third instar and fed old foliage, that numbers were too small to be of any significance in studies on development.

Daily records were kept of the emergence of male and of female adults from each lot. Generally, the female insects reared on old foliage from the fourth instar were, at the time of adult emergence, 14 days behind those reared on new foliage; the males were retarded eight days. In the lots started in the fifth instar, insects of both sexes reared on 1948 foliage took six days longer to complete development than those reared on 1949 foliage. In those reared on 1947 foliage, the females were further retarded in their development by another three days and the males by another two days. In the lots started in the sixth instar, a retardation of about one day occurred in both sexes of the insects reared on old foliage.

The following synopsis gives the mortality of insects taken from the field at different stages of development, and reared on 1948 and 1949 balsam fir foliage. All deaths recorded were from causes other than parasitization. Mortality increased with the amount of time that the larvae were forced to feed on old foliage.

| Instar at the time larvae were collected from the field | No. of insects failing to reach adult stage | |
|---|---|---|
| | Fed on 1948 foliage | Fed on 1949 foliage |
| III | 197 | 62 |
| IV | 125 | 46 |
| V | 119 | 59 |
| VI | 55 | 54 |

Of those insects collected in the field in the fifth instar and reared on the 1947 foliage, 110 died. The mortality did not differ from that which occurred in the lot collected at the same time and fed 1948 foliage.

In the field, mortality of larvae forced to feed on old foliage would probably be much higher. These larvae do a considerable amount of intra- and inter-tree wandering in search of new growth before resorting to feeding on old foliage; of those that abandon the host tree, many are undoubtedly lost.

An experiment was carried out in 1948 to measure the effect of the two foods, staminate flowers and needles, on survival after larval establishment. Five-inch to seven-inch branch tips with established populations were collected in the field. The larvae were counted on each tip. The tips were placed in lantern globe cages with the cut ends protruding through a floor into a container with water. Thirty-eight branches were set up on May 17, 18 with flower clusters and 20 without. They were re-examined on May 26, when pollen was shed in the field and the larvae began to migrate to the opening buds. On branches with staminate flowers, 92.5% of 298 larvae originally established in flowers had survived; on branches without flowers, 81.0% of the 239 larvae originally established in needles had survived. A chi-square test shows that the difference in survival rate (about 11%) is not significant. In the field few dead larvae were found in either flowers or needle mines. This does not mean that mortality is not high at this time; many larvae may be lost while migrating from the needles and flowers to the buds.

Oviposition experiments were carried out with the insects reared on new and old foliage in 1949. Pairs of newly emerged adults were placed in jars for mating. Females were always mated with males which had been reared on the same regimen. Females that had been observed in copula were placed in lantern globe cages with foliage, and oviposition was obtained from 63 mated females. The moths from the lots fed on old foliage laid fewer eggs than the moths from the lots fed on new foliage.

| Stage at which insects were collected from the field | Old foliage | | New foliage | |
|---|---|---|---|---|
| | No. of females | Average no. of eggs | No. of females | Average no. of eggs |
| IV | 0 | — | 13 | 185 ± 9 |
| V | 11 | 79 ± 10 | 20 | 144 ± 8 |
| VI | 11 | 84 ± 27 | 8 | 157 ± 10 |

The pupal weight of insects from the foregoing experiment was determined. Results for the lots taken from the field in the fourth, fifth, and sixth instars are shown in Table VI. Pupae of both sexes from populations reared on new foliage weighed significantly more than those reared on old foliage. The pupal weight of insects reared on old foliage from each of the fourth and fifth instars

TABLE VI

MEAN PUPAL WEIGHTS OF INSECTS COLLECTED IN THE FIELD AS FOURTH-, FIFTH-, OR SIXTH-INSTAR LARVAE AND REARED ON DIFFERENT FOODS UNTIL PUPATION

| Stage collected in field | Sex of pupae | Food provided during rearing | No. of pupae | Mean weight in grams |
|---|---|---|---|---|
| IV | Male | 1948 NF | 28 | .0380 ± .0018 |
| | | 1949 NF | 29 | .0546 ± .0066 |
| | Female | 1948 NF | 26 | .0447 ± .0020 |
| | | 1949 NF | 43 | .0855 ± .0024 |
| V | Male | 1947 F | 34 | .0338 ± .0071 |
| | | 1947 NF | 45 | .0303 ± .0012 |
| | Female | 1947 F | 36 | .0380 ± .0020 |
| | | 1947 NF | 39 | .0418 ± .0043 |
| | Male | 1948 NF | 64 | .0354 ± .0018 |
| | | 1949 NF | 79 | .0630 ± .0013 |
| | Female | 1948 NF | 45 | .0461 ± .0020 |
| | | 1949 NF | 60 | .0860 ± .0053 |
| VI | Male | 1948 NF | 64 | .0412 ± .0018 |
| | | 1949 NF | 74 | .0631 ± .0031 |
| | Female | 1948 NF | 62 | .0515 ± .0016 |
| | | 1949 NF | 67 | .0819 ± .0021 |

was significantly less than that of insects reared on old foliage from the sixth instar. This would indicate that the pupal weight was affected by length of time that the insects fed on old foliage. The female pupae ensuing from field-collected fifth-instar larvae fed 1947 foliage, weighed less than those from the same collection but fed 1948 foliage. Although the difference is not significant ($P = .08$), it suggests that the older the foliage, the lower the pupal weight. The source of the old foliage (flowering or nonflowering trees) did not influence the results.

There was a strong positive correlation between the weight of the pupae and the number of eggs deposited by the insects reared on old foliage from the fifth or sixth instar. There was no evidence of correlation between pupal weight and the number of eggs laid when the insects were reared on new foliage from the fourth, fifth, or sixth instar.

In 1948, the pupae ensuing from larvae collected in the field just prior to the shedding of pollen and reared on 1948 foliage in the laboratory, were weighed. The results are shown below.

| Source of larvae | Sex | Number of pupae | Mean weight, gm. |
|---|---|---|---|
| Flowers | Male | 39 | 0.0827 ± 0.0021 |
| Foliage | Male | 56 | 0.0785 ± 0.0020 |
| Flowers | Female | 48 | 0.1214 ± 0.0029 |
| Foliage | Female | 46 | 0.1137 ± 0.0022 |

Although in both sexes the mean pupal weight of insects originally from flowers was greater than the mean pupal weight of insects from foliage, the mean differences were not significant. Jaynes and Speers (5) dissected gravid females and found no evidence of a relationship between egg potential and the inclusion of pollen in the larval diet.

## Defoliation and Damage

Table VII gives the defoliation of the current year's foliage for typical flowering, typical nonflowering, and the intermediate category of balsam trees tagged for defoliation and mortality studies. All the plots, except Plot 6, were established in the year of first severe budworm defoliation (1946 for Plots 1, 2, 3, and 4; 1947 for Plots 5, 7, 8, and 9). Examination of foliage showed that in each of these Plots, defoliation on balsam fir had been less than 10% for the preceding years. Plot 6 was established in 1947 and it was estimated that defoliation on balsam fir had been approximately 75% of the current year's growth in 1946 and less than 10% in 1945. Table VII shows that flowering balsam trees were more heavily defoliated than nonflowering trees in the earlier stages of the infestation (Plots 1, 2, 3, and 4 in 1946; Plots 5, 7, 8, and 9 in 1947); and that in more advanced infestations, both types suffered severe defoliation (Plots 1, 2, 3, 4, and 6 in 1947; all the plots in 1948). Bess (2) mentions that flowering balsam fir trees were more heavily defoliated than the nonflowering trees in the Kabonga area of Quebec in 1945. The three Plots 2, 5, and 9 are the only ones where the nonflowering trees were not completely stripped of the current year's growth in any of the five years. A large percentage of the trees in Plots 2 and 9 are of the nonflowering type. In Plot 5, a number of nonflowering trees are shaded by poplar. In these three plots, the nonflowering trees were not subjected to as severe an influx of the later feeding instars dropping from the flowering balsam fir and the large white spruce trees as were the nonflowering trees in the other six plots.

In 1949, populations declined and defoliation was less severe, especially in Plots 1, 3, 4, and 6 where populations had been heaviest for the longest time. With the exception of Plot 4, the flowering trees exhibited a higher degree of defoliation than the nonflowering trees, although in 1949 the difference was not as striking as in the earlier stages of the infestation.

In 1950, the populations again reached high levels in all the areas under study. The current year's growth was either completely or almost completely destroyed on all balsam fir trees.

By 1950, a few small balsam fir trees had died as a result of defoliation in Plots 1 and 4, but none of the tagged trees had died in any of the plots, although some of the nonflowering trees were almost completely stripped of foliage in the areas where spruce budworm populations had been most active since 1946.

Because the flowering trees harbored higher populations and in the first years of infestation showed heavier defoliation than nonflowering trees, it might seem that the former type is more vulnerable to budworm attack.

TABLE VII

AVERAGE D.B.H., TOTAL HEIGHT, CROWN LENGTH, CROWN DIAMETER, AND DEFOLIATION OF THE CURRENT YEAR'S GROWTH OF TREES TALLIED IN THE VARIOUS PLOTS

| Plot No. | No. of trees | Tree type | Av d.b.h. | Av. total height | Av. crown length | Av. crown diam. | Av. per cent defoliation of the current year's growth | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 1946 | 1947 | 1948 | 1949 | 1950 |
| 1 | 22 | F | 7 | 47 | 26 | 9 | 95 | 100 | 100 | 25 | 100 |
| | 17 | NF | 5 | 30 | 20 | 9 | 50 | 100 | 100 | 10 | 95 |
| 2 | 12 | F | 8 | 41 | 20 | 12 | 50 | 95 | 100 | 90 | 100 |
| | 54 | NF | 6 | 30 | 24 | 10 | 25 | 75 | 95 | 90 | 95 |
| 3 | 24 | F | 9 | 58 | 39 | 12 | 95 | 100 | 100 | 75 | 100 |
| | 32 | NF | 5 | 35 | 22 | 10 | 75 | 100 | 100 | 75 | 100 |
| 4 | 21 | F | 8 | 66 | 35 | 8 | 90 | 100 | 100 | 50 | 100 |
| | 6 | I | 6 | 54 | 25 | 7 | 50 | 100 | 100 | 75 | 100 |
| | 17 | NF | 5 | 47 | 25 | 9 | 25 | 100 | 100 | 75 | 100 |
| 5 | 24 | F | 9 | 58 | 26 | 13 | | 25 | 100 | 100 | 100 |
| | 18 | I | 8 | 49 | 26 | 13 | | 10 | 100 | 95 | 90 |
| | 16 | NF | 6 | 40 | 31 | 11 | | 5 | 95 | 95 | 90 |
| 6 | 29 | F | 9 | 62 | 28 | 11 | | 100 | 100 | 75 | 100 |
| | 10 | I | 6 | 39 | 21 | 11 | | 100 | 100 | 50 | 100 |
| | 25 | NF | 8 | 49 | 26 | 14 | | 100 | 100 | 50 | 100 |
| 7 | 12 | F | 7 | 44 | 26 | 10 | | 75 | 100 | 100 | 100 |
| | 35 | I | 6 | 40 | 23 | 10 | | 50 | 100 | 95 | 100 |
| | 14 | NF | 5 | 33 | 17 | 11 | | 50 | 100 | 95 | 100 |
| 8 | 20 | F | 9 | 66 | 37 | 12 | | 25 | 100 | 100 | 95 |
| | 18 | I | 8 | 56 | 26 | 13 | | 10 | 100 | 100 | 90 |
| | 19 | NF | 7 | 41 | 23 | 14 | | 10 | 100 | 100 | 90 |
| 9 | 9 | F | 10 | 62 | 33 | 12 | | 50 | 100 | 95 | 95 |
| | 8 | I | 9 | 50 | 27 | 13 | | 25 | 100 | 90 | 75 |
| | 29 | NF | 5 | 28 | 19 | 10 | | 5 | 90 | 90 | 90 |

NOTE: F = *Flowering.* I = *Intermediate flowering.* NF = *Nonflowering.*

Indeed such is the case where the two physiological types do not occur intermixed. Isolated stands of nonflowering or physiologically immature balsam trees are less vulnerable to the budworm. However, balsam fir usually occurs in uneven-aged stands with the two types intermixed. As a result of the overflow of populations from centers of concentration on flowering trees, the smaller nonflowering trees are subjected to severe defoliation, and often succumb more quickly than the flowering trees. In 1947, the nonflowering trees in Plot 1 were not only stripped of the current year's growth but of two to four years' old foliage, owing to the overflow of fifth- and sixth-instar larvae from the flowering trees. The same conditions prevailed in Plots 3, 4, and 6 in 1948.

To illustrate to what extent the overflow of populations from the flowering trees affected the surrounding trees, seven black spruce trees averaging 6 in. d.b.h. and 33 ft. in height, were felled in 1947 in a stand of pure black spruce, 1000 ft. from Plot 7. Ten branches were taken along the crown of each tree. In a sample of 7233 terminal shoots, 13% showed some defoliation. Similarly five black spruce trees averaging 6 in. d.b.h. and 32 ft. in height, were taken within Plot 7. In a sample of 4186 terminal shoots, 64% were attacked. The difference in defoliation was due to large numbers of larvae dropping from the balsam fir and white spruce trees in the plot, and to the absence of such a source of larvae in the pure black spruce stand.

In 1947, on six nonflowering trees in Plot 6, 100% of the buds were attacked. In a small pocket of nonflowering balsam fir, 300 ft. away from the immediate influence of flowering trees, the average proportion of buds attacked on four trees was 80%. At the end of the feeding season in 1948 the same trees in the plot were completely defoliated, while those just outside the plot still retained a considerable amount of foliage.

Population and defoliation studies of advance balsam growth (trees 5 to 10 ft. high) in two different areas, further substantiate the fact that the overflow of populations from flowering trees affects the surrounding trees. One of the areas studied was in Plot 8, where the forest canopy was made up, for the most part, of budworm host tree species. The second one was at Jackfish Lake about four miles north of Plot 8, and closer to the center of the infestation. In this stand, the canopy was made up of jack pine, which was not infested, although heavily attacked spruce and balsam stands were only a few hundred feet away.

In each area, three 18-in. branch tips were taken from the top, middle, and lower parts of the crowns of 17 advance growth balsam fir trees. The terminals, attacked terminals, and insects were counted and defoliation was estimated for each branch.

| Location | No. of terminals | No. of attacked terminals | % Defol. | No. of pupae | No. of pupae per 100 terminals |
|---|---|---|---|---|---|
| Plot 8 | 1211 | 1061 | 61–70 | 153 | 12.6 |
| Jackfish Lake | 1106 | 301 | 0–10 | 9 | 0.8 |

Even though the advance balsam fir growth at Jackfish Lake was in the vicinity of a heavy infestation for one to two years longer than the advance growth balsam in Plot 8, the former was only lightly infested and defoliation was unimportant.

The cases just mentioned exemplify defoliation resulting from the overflow of larvae from flowering balsam fir trees at the time of the fifth and sixth instars. The overflow, in the form of widespread drift of the first- and second-instar larvae and to a certain extent of the ovipositing moths is far more difficult to measure, and far more important, as it is responsible for extensive distribution of populations.

## Summary and Conclusions

1. To determine the influence of flowering balsam fir trees on spruce budworm populations, investigations were conducted in Northwestern Ontario between 1946 and 1950.

2. Flowering balsam fir trees differ from nonflowering trees in a number of characteristics associated with physiological development, the more outstanding differences being a heavier and more frequent production of flowers, a larger number of buds per given length of branch, and a shorter shoot growth on flowering than on nonflowering trees. Generally, flowering trees occupy a more dominant position in the stand than nonflowering trees.

3. In studies of budworm populations on 176 flowering trees, and an equal number of nonflowering trees, in nine plots, eggs were generally found in greater abundance on the flowering trees. Larvae newly emerged from hibernation were more abundant on the flowering trees, both in absolute numbers, and also relative to the number of buds on the trees. Extensive wandering of the larvae in the later feeding stages resulted in a more random distribution of the populations; in the sixth larval instar and in the pupal stage, the populations in relation to the number of buds were similar on the flowering and the nonflowering trees.

4. Results of experiments and studies of the distribution of overwintering populations indicated that the first-instar larvae showed a preference for staminate flower cups as niches for the spinning of hibernacula.

5. On emergence in the spring, the second-instar larvae showed a strong preference for the staminate flowers as food. Where flowers were not available the larvae mined the needles of foliage of the most recent growth.

6. Larvae which fed on pollen during their early development had an advantage of about three days in their rate of development over larvae feeding exclusively on foliage. This advantage persisted throughout the developmental stages.

7. There appeared to be no difference in the survival and the fecundity of insects which fed during their early development on pollen and those that fed throughout their development on new foliage.

8. Insects fed on old foliage showed a higher rate of mortality, developed more slowly, and laid fewer eggs than insects fed on the current year's growth.

The degree to which survival and rate of development were affected depended on the length of time the insects fed on old foliage.

9. In the earlier stages of the infestation defoliation was more severe on flowering than on nonflowering trees. As populations increased, the currently produced foliage was exhausted before completion of larval development, large numbers of larvae abandoned the flowering trees and reached the nonflowering trees which were then severely defoliated.

It is concluded that flowering balsam fir trees are an important factor in providing conditions favorable to the increase of populations of the spruce budworm and thereby contribute to the initiation and spread of epidemics.

## Acknowledgments

The writer wishes to express his appreciation and thanks to Dr. M. L. Prebble, Officer-in-charge of the Forest Insect Laboratory in Sault Ste. Marie, whose advice and recommendations in the planning of the work, analysis of the data, and in the preparation of this paper were of very great value. Dr. C. E. Atwood, of the Department of Zoology of the University of Toronto, offered kindly criticism and many helpful suggestions. Grateful appreciation is expressed to J. M. Anderson, K. R. Elliott, L. M. Gardiner, J. L. Ladell, S. W. Lukinuk, A. H. Rose, and G. L. Woodbury, who assisted in the field studies. D. C. Anderson, Laboratory Photographer at Sault Ste. Marie, is also to be thanked for taking the photographs. A grant was received from the Ontario Research Commission during the academic year 1947–1948.

## References

1. Atwood, C. E. The feeding habits of the young spruce budworm larvae. Can. Entomol. 76 : 64-66. 1944.
2. Bess, H. A. Staminate flowers and spruce budworm abundance. Dom. Dept. Agr. Forest Insect Invest. Bi-monthly Progress Rept. 2 : 3-4. 1946.
3. Graham, S. A. and Orr, L. W. The spruce budworm in Minnesota. Univ. Minn. Agr. Expt. Sta., Tech Bull. 142. 1940.
4. Henson, W. R. The means of dispersal of the spruce budworm. PhD. Thesis, Yale University. 1950.
5. Jaynes, H. A. and Speers, C. F. Biological and ecological studies of the spruce budworm. J. Econ. Entomol. 42 : 221-225. 1949.
6. Morris, R. F. How old is a balsam tree? Forestry Chronicle, 24 : 108-110. 1948.
7. Snedecor, G. W. Statistical methods. Iowa State College Press, Ames, Iowa. 1946.
8. Swaine, J. M., Craighead, F. C., and Bailey, I. W. Studies on the spruce budworm *Cacoecia fumiferana* Clem. Can. Dept. Agr., Tech. Bull. 37 (n.s.). 1924.
9. Tothill, J. D. The passing of the balsam budworm in New Brunswick. Can. Forestry J. 15 : 306-307. 1919.
10. Wellington, W. G. Effects of radiation on the temperatures of insectan habitats. Sci. Agr. 30 : 209-234. 1950.
11. Wellington, W. G. and Henson, W. R. Notes on the effects of physical factors on the spruce budworm *Choristoneura fumiferana* (Clem.). Can. Entomol. 79 : 168-170; 195. 1947.

# DISEASE IN CODLING MOTH LARVAE PRODUCED BY SEVERAL STRAINS OF *BACILLUS CEREUS*[1]

By June M. Stephens[2]

## Abstract

This paper describes a bacteriological study of strains of *Bacillus cereus* Frankland and Frankland producing disease in codling moth larvae and an investigation of their pathogenicity. Strains of the bacterium were pathogenic to the codling moth by feeding or by injection.

## Introduction

The codling moth, *Carpocapsa pomonella* (L.), is one of the most persistent and destructive apple pests in orchards in most of Canada and the United States, and profitable production of apples requires that it be controlled by chemical and biological methods. In early attempts in Canada to use the biological method, parasitic insects were introduced from Europe. The propagation and release of these parasites was carried out by officers of the Dominion Parasite Laboratory, Belleville, Ont. In 1947 a disease appeared among codling moth larvae in stock at the Laboratory. This disease seriously interfered with propagation of the parasites, for which the codling moth larvae served as host material. A study of the disease was begun in order to check its spread in the laboratory, to facilitate maximum parasite production, and to consider the utilization of this disease as an agent of biological control. The disease was found to be caused by several strains of *Bacillus cereus* Frankland & Frankland. There is little mention in the literature of bacteria associated with the codling moth. Steinhaus (7) stated that the fireblight organism, *Erwinia amylovora* (Burrill) Winslow *et al.* has been found associated with the codling moth. Apparently this disease in orchards is sometimes transmitted by the codling moth. Boyce (1) referred to an unidentified disease prevalent in codling moth larvae collected from several orchards in the Niagara district. Since a strain of *B. cereus*, described in this paper, was isolated from diseased larvae collected in the same district, the disease mentioned by Boyce may have been caused by the same bacterium. A strain of *Serratia marcescens* Bizio will also produce disease in codling moth larvae.

## Isolation and Description of Organisms

The disease present in the laboratory stock at Belleville was noted particularly among overwintering larvae held in corrugated cardboard strips in the storage rooms. Bucher (unpublished data) isolated four strains of a

[1] *Manuscript received September 26, 1951.*

*Contribution 2881, Division of Entomology, Science Service, Department of Agriculture, Ottawa, Canada. Based on a thesis presented in August, 1949, to Queen's University, Kingston, Ont., in partial fulfillment of the degree of Master of Arts.*

[2] *Agricultural Research Officer, Biological Control Investigations Laboratory, c/o Department of Bacteriology, Queen's University, Kingston, Ont.*

bacterium from diseased overwintering larvae that had been obtained from widely scattered sources in Canada and the United States. He made a preliminary examination of the bacteria and established their pathogenicity for overwintering codling moth larvae. In addition to the four original strains, the writer has isolated seven strains of the same organism and a strain of a closely related organism, *Bacillus megatherium* De Bary. Table I lists the strains and their sources.

TABLE I

SOURCES OF CM STRAINS OF *Bacillus cereus*

| Group designation | Source of group | Strains | Source of each strain |
|---|---|---|---|
| CM 1 | Overwintering larvae from various localities in Canada and U.S.A. | CM 1-1 | United States (particular area unknown) |
| | | CM 1-2 | Niagara Peninsula |
| | | CM 1-3 | British Columbia |
| | | CM 1-4 | Indiana |
| CM 2 | External surface of apples | CM 2-1 | Morris Orchard, Bloomfield, Prince Edward County, Ont. |
| | Immature codling moth larvae | CM 2-2 | Morris Orchard, Bloomfield, Prince Edward County, Ont. |
| CM 3 | External surface of apples on neglected trees | CM 3-1 | Barriefield, Ont. |
| | | CM 3-2 | Seeley's Bay, Ont. |
| | | CM 3-5 | Chaffey's Locks, Ont. |
| | | CM 3-8 | Lombardy, Ont. |
| | | CM 3-9 | Carsonby, Ont. |
| M 5 | Field-collected diseased codling moth | CM 5-1 | Platt's orchard, Vineland Station, Ont. |

The following description is common to all strains except where differences between strains are noted.

*Morphology*

Rods, 0.9 to 1.4$\mu$ by 3.0 to 7.0$\mu$; average width 1.1$\mu$, average length 5.0$\mu$. Sides of rods parallel and ends slightly rounded (Fig. 1); occurring singly and in chains of 5 to 10 bacteria; motile by 10 to 12 peritrichous flagella. Capsules not demonstrated. Spores central to subterminal in position, causing little or no bulging of the cell wall; oval, the average size being 1.0 by 1.3$\mu$; few spores appearing on agar plates after 24-36 hr. growth. Gram positive; staining uniformly; old cultures showing tendency to stain Gram negative.

*Cultural Characteristics*

*Colony Formation on Nutrient Agar*

Roughly circular, 2 to 5 mm. in diameter after 24 hr. incubation; surface rough, finely granular, and low convex; friable consistency; adherent to medium; emulsification rather difficult. White in color, opaque and densest

at the center. Edge irregular. Varying from rough to smooth type of colony; variation reversible. Colony form on nutrient agar (Fig. 2). All strains readily grown on nutrient agar, but more luxuriantly on heart infusion agar.

*Growth on Agar Slants*

Abundant, white, faintly smooth surface; confluent and adherent to the medium.

*Growth on Other Media*

On blood agar, colonies subcircular to circular; average diameter 5.0 mm.; colonies dark gray with finely granular matlike surface; sometimes wrinkled. Beta-hemolysis observed in 24 hr.; hemolysis extremely pronounced after 48 hr., but less so in CM 3-1 than in other strains.

On potato slants, growth wrinkled and membranous, creamy white; almost indistinguishable from surface of potato.

In nutrient broth, growth resulting in medium to heavy turbidity; slight granular deposit sometimes present; a pellicle that sinks to the bottom of test tube sometimes formed.

*Physiological Characteristics*

*Oxygen Requirements*

Optimum growth occurs in presence of free oxygen although organisms will grow under anaerobic conditions. These bacteria can be classed as facultative anaerobes.

*Temperature Range*

All strains are capable of growing in a range of 12° to 43° C.; six with a maximum of 48° C. Optimum growth at 30° C. (Fig. 3).

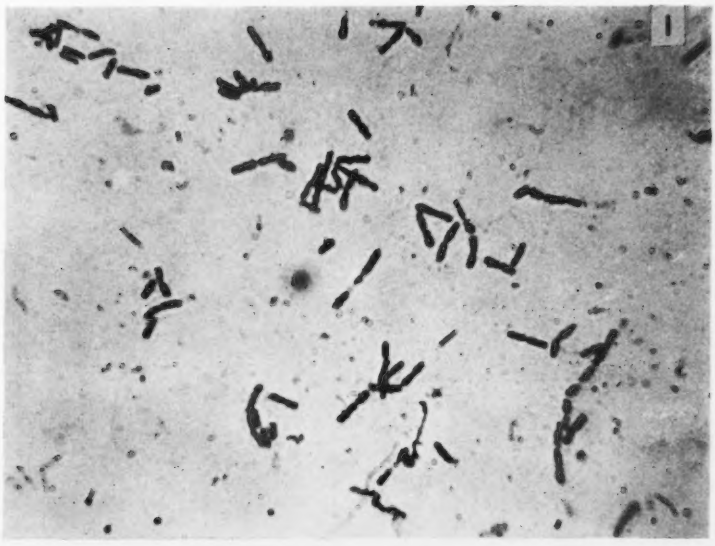
It is obviously important to know the temperature range of a bacterium that produces disease in an insect, particularly if the bacterium is being considered as a possible agent of biological control. As the codling moth overwinters in the larval stage, it is to be expected that an organism affecting it in this stage would be capable of survival and growth at low temperature. Strains of *B. cereus* fulfill this condition, since they survive temperatures lower than 12° C.

*Effects of pH on Growth*

Strains of these organisms grow over a pH range of 5.0 to 8.0. At pH 5, growth is slight and appears shiny as compared with the dull surface of colonies grown at the optimum pH of 7.2 to 7.6.

Organisms were grown in glucose proteose-peptone broth with an original pH of 6.8. After 24 hr. growth all strains had rendered the medium acid in the range of pH 5.1.

The pH of the gut of the codling moth was in the range of 7.5 to 8.0. This range is favorable for the growth of *B. cereus*.



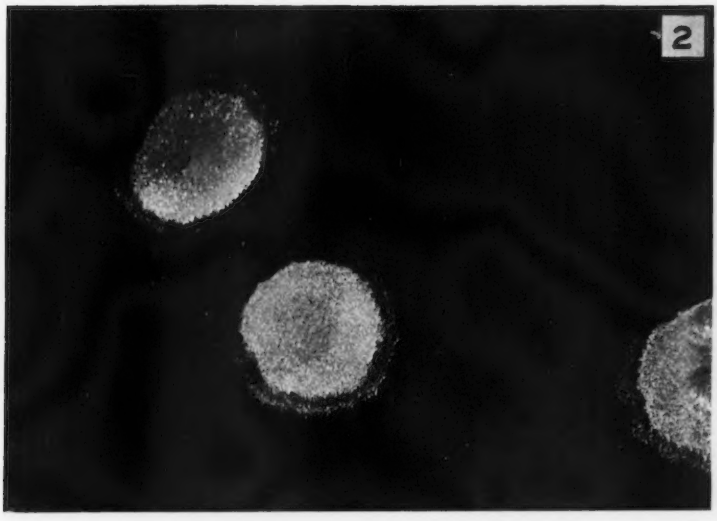




FIG. 1. *B. cereus*, 24-hr. culture; smear stained with Gram stain, showing rods with spores. Magnification approx. 1000×.

FIG. 2. Colonies of *B. cereus* after 48 hr. growth on nutrient agar. Magnification 3×.

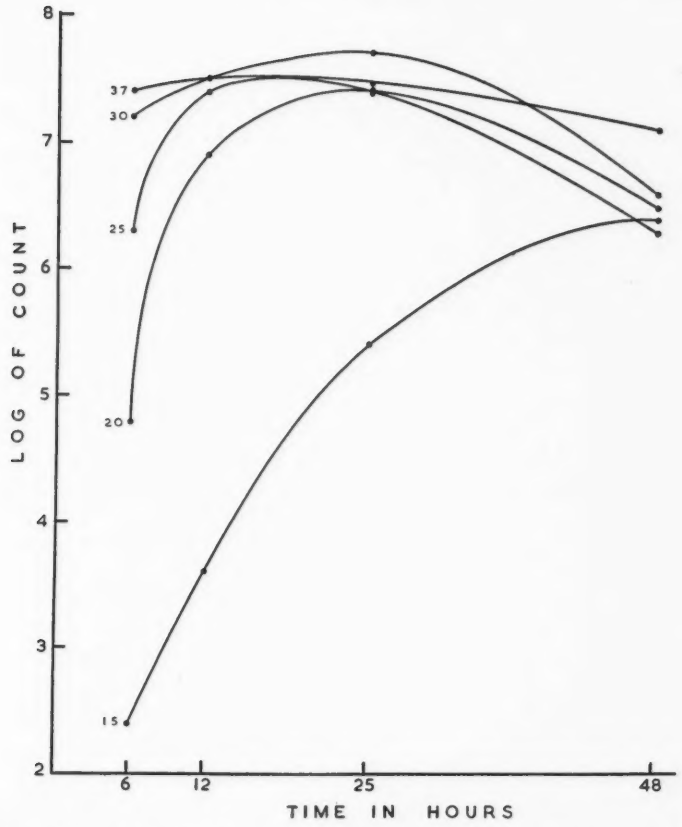



FIG 3. Typical growth rate of a CM strain of *B. cereus* over a temperature range of 15-37° C.

*Resistance and Survival*

Vegetative forms of this bacterium withstand 60° C. (moist heat) for two hours and spores are killed only after heating to boiling for two hours. Spores have survived at a constant relative humidity of 80% at a temperature of 25° C. for two years and also for two years in powdered peat, prepared by a method of Lochhead and Thexton (3).

*Growth Rate*

Growth rates were determined for cultures in nutrient broth at temperatures of 15° to 37° C. (Fig. 3). Counts of viable organisms were determined by the drop-plate method of Reed and Reed (4). The growth is relatively slow at 15° C. and increases with increasing temperature to a maximum rate in the 30° and 37° C. range.

TABLE II

DIFFERENCES IN BIOCHEMICAL REACTIONS OF STRAINS OF *B. cereus*

| | CM 1-1 | CM 1-2 | CM 1-3 | CM 1-4 | CM 2-1 | CM 2-2 | CM 3-1 | CM 3-2 | CM 3-5 | CM 3-8 | CM 3-9 | CM 5-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mannitol | — | — | — | — | — | — | A | — | — | — | — | — |
| Sucrose | A | A | A | — | — | A | A | A | — | A | — | A |
| Xylose | — | — | — | — | — | — | A | — | — | — | — | — |
| Galactose | — | — | — | — | — | — | A | — | — | — | — | — |
| Arabinose | — | — | — | — | — | — | A | — | — | — | — | — |
| Raffinose | — | — | — | — | — | — | A | — | — | — | — | — |
| Mannose | A | A | A | — | — | A | A | — | — | — | — | A |
| Milk | Pept. | Pept. | Pept | Pept | Pept | Pept. | Clot | Pept. | Pept. | Pept. | Pept. | Pept. |

NOTE: *Acid formed, A.*
*Peptonization, pept.*
*Reaction negative, —.*

*Biochemical Reactions*

The strains of the organisms tested showed some variability in their biochemical reactions. All strains produced acid without gas from glucose, maltose, fructose, trehalose, and dextrin. No acid was produced by any of the strains in lactose, dulcitol, rhamnose, sorbitol, adonitol, inositol, glycerol, or inulin.

Acetylmethylcarbinol is produced only under certain conditions (6, p. 12). With the strains tested it was possible to obtain a positive test only when the Voges–Proskauer medium was sterilized by filtration. Methyl red is negative to positive. Starch is hydrolyzed, indol not formed, hydrogen sulphide negative, and methylene blue reduced. Citrates are utilized as sole source of carbon and nitrates reduced to nitrites. Reactions in which some variability occurred are listed in Table II.

*Antigenic Properties*

To investigate the antigenic relationship of strains, rabbits were immunized with six doses of heat-killed vegetative organisms followed by six doses of living broth cultures at four-day intervals. Dosage in each case was increased from 0.1 to 1.0 ml. Agglutination tests were carried out with an antigen prepared by treating a 24 hr. broth culture with 0.2% formalin and then re-incubating for 48 hr. The reactions were carried out at 52° C. for two hours and final readings were made after the tubes had stood in the cold-room for 12 to 16 hr. Agglutination was light and of the granular type. Table III shows the results with the eight strains tested.

TABLE III

AGGLUTINATION REACTIONS OF SEVERAL CM STRAINS OF *B. cereus*

| Antigen | Antiserum titer | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CM 1-1 | CM 1-2 | CM 1-3 | CM 1-4 | CM 2-1 | CM 2-2 | CM 3-1 | CM 3-2 |
| CM 1-1 | 1 : 320 | 1 : 80 | 1 : 320 | 1 : 80 | 1 : 80 | 1 : 160 | 1 : 10 | 1 : 320 |
| CM 1-2 | 1 : 80 | 1 : 320 | 1 : 20 | 1 : 10 | 1 : 80 | 1 : 160 | 1 : 10 | 1 : 80 |
| CM 1-3 | 1 : 320 | 1 : 80 | 1 : 640 | 1 : 320 | 1 : 160 | 1 : 320 | 1 : 10 | 1 : 80 |
| CM 1-4 | 1 : 20 | 1 : 10 | 1 : 320 | 1 : 320 | 1 : 160 | 1 : 80 | 1 : 10 | 1 : 80 |
| CM 2-1 | 1 : 20 | 1 : 80 | 1 : 160 | 1 : 320 | 1 : 320 | 1 : 320 | 1 : 10 | 1 : 320 |
| CM 2-2 | 1 : 320 | 1 : 320 | 1 : 320 | 1 : 80 | 1 : 80 | 1 : 320 | 1 : 20 | 1 : 80 |
| CM 3-1 | 1 : 20 | 1 : 20 | 1 : 20 | 1 : 10 | 1 : 10 | 1 : 20 | 1 : 320 | 1 : 20 |
| CM 3-2 | 1 : 20 | 1 : 80 | 1 : 20 | 1 : 20 | 1 : 20 | 1 : 80 | 1 : 20 | 1 : 320 |

Little is known about the antigenic structure of aerobic spore-forming bacilli. Wilson and Miles (9, p. 840) stated that these bacteria form a group, the members of which are closely related antigenically. The results obtained

in this series of agglutinations suggest that most of the CM strains possess common antigens. Strain CM 3-1, differing biochemically from the other 11 strains, showed cross-agglutination at much lower titers than did most of the strains. The value of agglutination reactions in the classification of spore-forming aerobic bacilli is still indefinite. Sievers and Zetterberg (5) stated that antisera are often strongly agglutinated by heterologous strains. Whether the cross-agglutinations of CM strains indicates closely related strains or is merely a reaction between heterologous strains has not been definitely established. The results obtained show that CM strains are not good antigens.

*Identification*

On the basis of morphological and cultural characteristics, the 12 organisms described herein appear very similar. However, CM 3-1 shows enough differences in its biochemical and antigenic properties to be considered a distinct species. It closely resembles *Bacillus megatherium* De Bary (2, 6), but may more properly fit into the *B. cereus* – *B. megatherium* group of intermediates described by Smith *et al.* (6). The remaining 11 cultures most closely resemble *B. cereus* (2, 6).

## Determination of Pathogenicity

The pathogenicity of the CM strains was determined for warm-blooded animals and for the codling moth. Pathogenicity for the codling moth was determined by means of inoculation and by feeding.

*Pathogenicity for Animals*

With the exception of *B. anthracis* Cohn, which Smith (6) considers the virulent variety of *B. cereus*, most of the Gram-positive aerobic spore-forming bacilli show little pathogenicity for mammals (9). The CM strains produced no effects on guinea pigs or rabbits when 1 ml. of a 24 hr. broth culture was injected intramuscularly. However, 1 ml. of an undiluted 24 hr. broth culture injected intraperitoneally caused the death of a white mouse in 6 to 48 hr.

*Pathogenicity for the Codling Moth*

A laboratory culture of the codling moth for use in pathogenicity determinations was maintained for several generations. The insects were reared on apples from the egg to the adult stage.

*Injections*

Injection serves as a good screening method for determining the possible pathogenicity of a bacterium. Also, an approximate known number of bacteria can be introduced into the insect and thus the minimum number necessary to kill the insect can be determined. Mature larvae were used in all injection experiments. The larva was grasped lightly between the thumb

and forefinger and 0.01 ml. of bacterial suspension was introduced into the side of the larva in the area of the fifth abdominal segment by means of a $\frac{1}{4}$ ml. syringe and a 30 gauge needle.

As a control, larvae were injected with 0.01 ml. of an undiluted culture of a Gram-positive coccus isolated from a normal larva of the eastern tent caterpillar. Since no deaths resulted from the injection of this organism, apparently no serious mechanical injury occurred because of the injection of a large number of nonpathogenic bacteria (Table IV).

TABLE IV

MORTALITY OF CODLING MOTH LARVAE RESULTING FROM INJECTION WITH CM STRAINS OF *B. cereus*

| Strain | Approximate number of bacteria injected | Number of deaths out of 10 |
|---|---|---|
| Bacterial control | $4.4 \times 10^5$ | 1 |
| CM 1-1 | $1.6 \times 10^5$ | 10 |
| | $1.6 \times 10^3$ | 10 |
| | $1.6 \times 10$ | 4 |
| CM 1-2 | $2.2 \times 10^5$ | 10 |
| | $2.2 \times 10$ | 8 |
| CM 1-3 | $2.4 \times 10^5$ | 10 |
| | $2.2 \times 10$ | 8 |
| CM 1-4 | $1.4 \times 10$ | 10 |
| CM 2-1 | $0.6 \times 10$ | 9 |
| CM 2-2 | $0.7 \times 10$ | 2 |
| CM 3-1 | $0.6 \times 10$ | 7 |
| CM 3-2 | $0.8 \times 10$ | 9 |
| CM 3-5 | $0.7 \times 10$ | 7 |
| CM 3-8 | $0.5 \times 10$ | 10 |
| CM 3-9 | $1.0 \times 10$ | 10 |
| CM 5-1 | $0.6 \times 10$ | 7 |

Symptoms of infection were first evidenced by immobility and darkening of larvae. Death occurred in 24 to 48 hr., at which time larvae were black, soft, and shrunken.

Of the 12 strains of CM series of cultures, tested by injection of 5 to 25 bacteria into mature larvae, 10 strains killed 70 to 100% (Table IV). Positive evidence that the injected organisms produced infection was obtained by culturing all dead larvae. The injected organism was recovered in pure culture from all larvae that died after treatment.

*Feeding*

Larvae were reared on apples, infected with CM strains, and examined at regular intervals to determine the effects of feeding *B. cereus* organisms. The procedure was as follows:—

A flap of approximately 1 sq. cm. in area was cut in the side of the apple and a bit of the pulp scraped out. A drop of bacterial culture was placed in the depression and allowed to soak in. A larva was placed in the hollow and the flap of skin sealed in position with a small amount of melted paraffin wax. In this way, the larva was forced to eat its way through the culture-soaked apple.

This method permitted the following routes of infection: (*a*) oral, (*b*) respiratory, and (*c*) integumentary. The main route of infection was apparently by mouth, as most of the larvae that died had eaten their way partially through the apple. This method did not make it possible to determine the approximate number of bacteria necessary to infect a larva. However, the larvae were exposed to only one drop of culture, approximately $10^6$ bacteria, and probably consumed less. The number of viable organisms in the apple rapidly diminished. Therefore, to become infected, a larva had to eat its way through the culture-soaked apple very soon after addition of the culture.

All but two of the control insects, placed in apples soaked with sterile broth, reached maturity.

Any larvae that were susceptible to infection with CM strains showed symptoms in 24 to 72 hr. The first symptom of infection was sluggishness accompanied by the appearance of brown spots on the integument. The larvae became practically motionless and the brown color spread and covered the whole integument. At time of death, larvae were soft and flaccid and almost black. The internal organs were broken down and viscous fluid seeped out of the body wall. In every case the fluid yielded an almost pure culture of the bacterium fed. The relationship of bacteria to the disease was established by fulfilling Koch's postulates.

All larvae used in these feeding trials were in the late fourth or the early fifth instar. Table V indicates results obtained from feeding experiments. Most of the larvae exposed to infection developed the disease and died; control larvae handled in the same manner, but not exposed to infection, did not die. The mortality rate ranged from 20 to 75%. Most strains of *B. cereus* isolated from insects showed a greater degree of virulence than did strains isolated from apples. Insect passage appeared to increase the virulence of strain CM 1-4. The original bacterium killed 40% of exposed larvae and after three passages 100% mortality was obtained. In view of the nature of the experimental procedure, it is evident that this series of bacteria has a high level of virulence for codling moth larvae.

TABLE V

MORTALITIES OF 4TH- AND 5TH-INSTAR LARVAE OF THE CODLING MOTH IN APPLES INFECTED WITH VARIOUS STRAINS OF *B. cereus*

| Strain | Larvae exposed to infection | | |
|---|---|---|---|
| | No. tested | No. dead | % dead |
| CM 1-1 | 20 | 13 | 65 |
| CM 1-2 | 20 | 14 | 70 |
| CM 1-3 | 20 | 15 | 75 |
| CM 1-4 | 40 | 26 | 65 |
| CM 2-1 | 20 | 14 | 70 |
| CM 2-2 | 15 | 5 | 33 |
| CM 3-1 | 15 | 7 | 47 |
| CM 3-2 | 15 | 7 | 47 |
| CM 3-5 | 15 | 3 | 20 |
| CM 3-8 | 15 | 8 | 53 |
| CM 3-9 | 20 | 6 | 30 |
| CM 5-1 | 15 | 8 | 53 |
| Total | 230 | 126 | 55 |
| Control | 130 | 2 | 1.5 |

## Discussion

*Bacillus cereus* was first observed in association with the codling moth at the Dominion Parasite Laboratory, Belleville, Ont. Diseased codling moth larvae contained large quantities of this organism. Strains of the bacterium were isolated from both the codling moth and apples from localities throughout the United States and Canada. It would appear that *B. cereus* is frequently associated with apples and the codling moth in nature.

The organism shows a difference in pathogenicity for mammals and for the codling moth. *B. cereus* has a very low pathogenicity for mammals but less than 100 bacteria cause the death of the codling moth when they are injected into the haemocoele. When a relatively low number of organisms are fed to the codling moth it appears that the bacteria are capable of invading the haemocoele and causing the death of the insect. Pathogenicity of spores alone has not been determined.

Strains of *B. cereus* are possible agents of biological control of the codling moth because they are moderately pathogenic for codling moth larvae in the laboratory. They can be produced readily in the laboratory on artificial

media and survive for a long period of time in the spore stage. They can be applied as sprays by commercial sprayers or can be incorporated in a peat dust carrier and applied to apple trees by commercial dusting apparatus.

Steinhaus (8) has experimented with the use of a similar bacterial spore former (*Bacillus thuringiensis*) in the control of the alfalfa caterpillar. He found that this organism gave a faster control than the virus disease although field mortality was not as great. Smith (6) believes that *B. thuringiensis* is merely a strain of *B. cereus*. In this laboratory Steinhaus' strain of *B. thuringiensis* was found to be biochemically and culturally identical with strains of *B. cereus* isolated from the codling moth. However, under certain cultural conditions, *B. thuringiensis* produces spores which lie obliquely in the sporangium, but oblique spores have never been observed in the strains of *B. cereus* obtained from the codling moth.

Trials are in progress to determine the extent to which *B. cereus* may be used as an agent in biological control of the codling moth in apple orchards.

## Acknowledgments

The author wishes to thank Dr. G. B. Reed and Dr. A. S. West of Queen's University for direction in this work, and Dr. G. E. Bucher of the Biological Control Investigations Laboratory, Kingston, for permission to refer to work conducted by him and for assistance with this manuscript.

## References

1. Boyce, H. R. Biological control of the codling moth in Ontario. Ann. Rept. Entomol. Soc. Ontario, 71 (1940) : 40-44. 1941.
2. Breed, R. S., Murray, E. G. D., and Hitchens, A. P. Bergey's manual of determinative bacteriology. 6th ed. The William and Wilkins Company, Baltimore. 1948.
3. Lochhead, A. G. and Thexton, R. H. Growth and survival of bacteria in peat. I. Powdered peat and related products. Can. J. Research, C, 25 : 1-13. 1947.
4. Reed, R. W. and Reed, G. B. "Drop-plate" method of counting viable bacteria. Can. J. Research, E, 26 : 317-326. 1948.
5. Sievers, O. and Zetterberg, B. A preliminary investigation into the antigenic characters of the sporeforming aerobic bacilli. J. Bact. 40 : 45-57. 1940.
6. Smith, N. R., Gordon, R. E., and Clark, F. E. Aerobic mesophilic sporeforming bacteria. U.S. Dept. Agr. Misc. Pub. 559. 1946.
7. Steinhaus, E. A. Insect microbiology. Comstock Publishing Company, Inc., Ithaca, N.Y. 1946.
8. Steinhaus, E. A. Possible use of *Bacillus thuringiensis* Berliner as an aid in the biological control of the alfalfa caterpillar. Hilgardia, 20 : 359-381. 1951.
9. Wilson, G. S. and Miles, A. A. Topley and Wilson's principles of bacteriology and immunity. Vols. 1 and 2, 3rd ed. Edward Arnold and Co., London. 1947.

# DIETARY FAT AND TEMPERATURE TOLERANCE OF GOLDFISH[1]

By William S. Hoar[2] and Merva K. Cottle[3]

## Abstract

Resistance of goldfish to low as well as to high temperatures may be changed by feeding diets containing different fats in high concentration. There is no evident relationship between melting point of the natural fats (pilchard oil, herring oil, and lard) and tolerance to cold. Hydrogenation of pilchard oil used in the diets reduces resistance of goldfish to both low and high temperatures. Fish fed on hydrogenated cottonseed oil show a similar reaction to high temperatures. These findings are contrary to the theory that increased thermal resistance is associated with higher melting point fats. It is suggested that the presence of double bonds in the lipids is important in maintaining the integrity of the plasma membrane.

## Introduction

Hoar and Dorchester (6) have shown that the heat tolerance of goldfish (*Carassius auratus*) can be markedly changed by feeding different types of fat in high concentration. The diets used contained 30% lard, herring oil, or pilchard oil. Fish feeding on the hard fat withstood higher temperatures than those feeding on the liquid fats. The upper lethal temperatures were thus related in a general way to the melting point or the degree of unsaturation of the body fats in accordance with certain theories of heat death (5). There was, however, no precise correlation between the degree of unsaturation of the body fats and the ability to withstand high temperatures. The fats used in these experiments were from different animals and most certainly varied in many properties other than their melting points. They may not, therefore, be suitable to test the relationship under consideration.

Similar experiments have consequently been carried out using a series of hydrogenated pilchard oils. The process of hydrogenation reduces the degree of unsaturation and produces fats with higher melting points. Although other changes in properties may be expected, the fats will differ primarily in their degree of unsaturation and should provide precise information on the relationship between unsaturation of body fats and thermal tolerance.

In addition to the hydrogenated pilchard oils, diets containing cottonseed oil and its hydrogenated product "Crisco" were studied. In another series fish were fed on the original diets of lard, herring oil, and pilchard oil to check the upper lethal temperatures and determine differences in the lower lethal temperatures produced by feeding fats in high concentration. The effect of diet on the lower lethal temperatures was not previously recorded.

[1] *Manuscript received September 27, 1951.*
*Contribution from the Department of Zoology, University of British Columbia, Vancouver, B.C., with financial assistance from the National Research Council of Canada.*

[2] *Professor of Zoology and Fisheries.*
[3] *Graduate Student.*

## Materials and Methods

The study is based on approximately 1500 goldfish (initial weights 10 to 15 gm.) obtained from the Goldfish Supply Co., Stouffville, Ont. They were divided into lots of 100 fish and maintained in separate aquaria with water thermostatically controlled at 20° ± 1° C. Fish for each series of experiments were purchased at one time and were presumably subjected to similar conditions prior to shipment.

Methods for the preparation of the high fat diets and for the feeding and care of the goldfish have been described (6). Fats used are listed in Table I, together with the iodine value of the original oil, of the ether extract of the diet prepared therefrom, and of the oil extracted from the fish at the close of the feeding experiment. The duration of the feeding experiment is also given in Table I, as well as the moisture and fat content of the goldfish tissues at the end of experimentation. Experiments were carried out in four series as indicated.

The hydrogenated pilchard oils were prepared by Dr. Lyle Swain of the Pacific Fisheries Experimental Station. The hydrogenation was carried out at 25 lb. pressure and 180° C. in the presence of 0.26% nickel as found in "Selectol A". "Selectol A", a hardened fat containing 16.44% nickel, is a hydrogenation catalyst prepared by E. F. Drew & Co., Inc. The nickel was removed, after hydrogenation, by filtration followed by washing with dilute sulphuric acid and water. The final product contained 1.4% of the hardened fat from the "Selectol A" and less than 0.05 mgm. nickel per gram of fat. We are indebted to Dr. Swain for the preparation of these fats and for the details of their preparation as given here.

In determining thermal resistance, all the fish of any series were tested at one time in the same large tank divided by plastic netting into an appropriate number of compartments. Unless otherwise indicated in the figures, resistance to cold was studied at 2.2 ± 0.3° C.; resistance to heat at 36 ± 1° C. Approximately half of the fish on any diet were tested at each of these temperatures. Fish which exhibited a negative response when prodded with a glass rod were considered dead. Tests for heat resistance were terminated after 24 hr.; for cold resistance after 48 hr.

Half of the fish which died at low temperatures were used for moisture determination; the remainder for oil analyses. After wiping off excess moisture the whole fish was placed in a test tube and dried to constant weight (usually about four days) in an oven at 95° C. This procedure was modified in Series 3 (Table I) by mincing fish in a Waring Blendor and drying samples to constant weight in an oven. Fish which died in each 12-hr. period of exposure were treated separately in this and other analyses. Since, however, differences were not evident in the groups dying in the four periods the results presented here are averages for the four groups. Oil was extracted from tissues of goldfish macerated in the Waring Blendor. Samples were dried *in vacuo* until crisp at 50°-60° C. and extracted with peroxide-free ethyl ether in the

A.S.T.M. extractors at 37° C. Ether was subsequently removed, *in vacuo*, at 37° C. and the oil placed in a desiccator for final drying and weighing. Iodine values were determined by Wijs' method.

## Results

Goldfish readily consumed the high-fat diets. Fish receiving lard or pilchard oil in any form were in excellent condition throughout the entire feeding period. Herring oil produced a mortality of approximately 17% during the first week. Thereafter, the fish recovered and although they were excitable, fat accumulated in the tissues (Table I). At the time of the thermal tests, these fish were evidently in as good condition as the other fish of the series. Goldfish did not respond well to the diets of cottonseed oil. During the 32-day period there was a 26% mortality of the fish on cottonseed oil, and 19% of those on "Crisco". It is evident from Table I that these fish stored little fat during the period. The fat content for a group of nine control fish held at 20° C. averaged 1.17%. All fish fed on high fat diets showed values substantially higher than this. With the exception of those fed on cottonseed products the values were two to five times as high as the control value. It is perhaps interesting to note here that pigs fed on menhaden oil (2) and pigs fed on cottonseed oil (3) have shown peculiar nervous symptoms.

TABLE I

DIETS USED AND THE EFFECT OF THESE ON THE TISSUES OF GOLDFISH. SERIES 4 WAS TESTED ONLY FOR RESISTANCE TO HIGH TEMPERATURE

| Series | Diet | Iodine values | | | Duration of experiment (days) | | Tissue analyses | |
|---|---|---|---|---|---|---|---|---|
| | | Original fat | Oil extracted from: | | Cold test | Heat test | Moisture, % | Lipid, % |
| | | | Diet | Fish | | | | |
| 1 | Natural fats | | | | 48 | 52 | | |
| | Lard | 65 | 64 | 87 | | | 74.9 | 5.4 |
| | Herring oil | 134 | 114 | 109 | | | 75.0 | 5.0 |
| | Pilchard oil | 172 | 147 | 135 | | | 74.6 | 5.1 |
| 2 | Hydrogenated pilchard oils | | | | 60 | 65 | | |
| | *A* | 64 | 64 | 90 | | | 76.6 | 3.1 |
| | *B* | 92 | 84 | 96 | | | 76.8 | 3.3 |
| | *C* | 138 | 92 | 113 | | | 77.3 | 2.9 |
| | *D* | 150 | 103 | 106 | | | 79.6 | 2.0 |
| 3 | Hydrogenated pilchard oils and lard | | | | 50 | 49 | | |
| | *A* | 65 | 65 | 98 | | | 77.6 | 4.3 |
| | *B* | 92 | 85 | 101 | | | 77.2 | 3.5 |
| | *C* | 150 | 132 | 105 | | | 77.5 | 4.3 |
| | Lard | 64 | 64 | 88 | | | 77.3 | 4.3 |
| 4 | Cottonseed oil | | | | — | 32 | | |
| | Oil | 106 | 100 | 109 | | | — | 1.5 |
| | "Crisco" | 77 | 78 | 94 | | | — | 1.9 |

Table I shows that each series of diets provided a range in unsaturation, as shown by iodine values of the body fats. Some groups responded better than others but, in each case, definite differences are evident.

*Low Temperature Tolerance*

The three natural fats (pilchard oil, herring oil, and lard) produced marked differences in resistance of goldfish to low temperatures. Data for two experiments (Figs. 1 and 2) show that the fish fed pilchard oil are most susceptible and that those fed on herring oil resist low temperatures best. In the principal series (Fig. 1) there is a difference of over 12 hr. between the times for 50% mortality in these two groups.

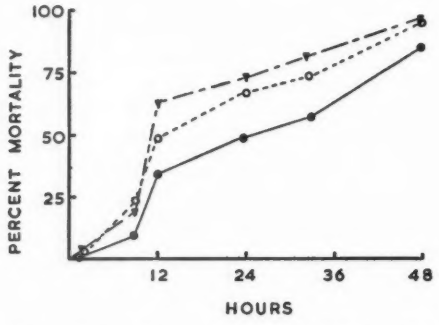



FIG. 1. Low temperature tolerance for goldfish fed on pilchard oil ——·——·——; herring oil ————————; and lard – – – – –. Temperature fell to 0.7° C. between Hours 9 and 12 then gradually rose to the constant test temperature of 2.2 ± 0.3° C. by 24 hr.

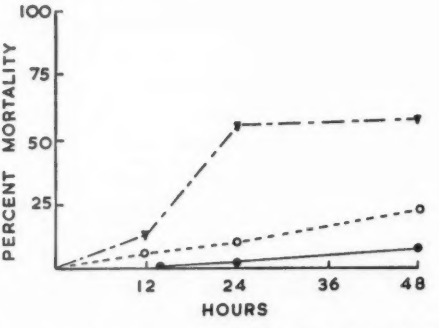



FIG. 2. Low temperature tolerance for goldfish fed pilchard oil ——·——·——; herring oil ————————; and lard – – – – – – –. Test temperature fell gradually from the initial value of 4.5° C. to 3.0° C. at 12 hr. and 2.0° C. at 24 hr., then rose gradually to 4.0° C. at 48 hr. Data obtained by E. W. Burridge.

The resistance of goldfish to low temperatures is decreased by hydrogenation of the pilchard oil used in the diets. Again, considering the time required to bring about 50% mortality in the samples, it is evident that a difference of

about 20 hr. in lethal time is associated with a change of 39 points in the iodine value (64 to 103) of their body fats (Fig. 3). The order was the same in the second test although the mortality was not as high (Fig. 4).

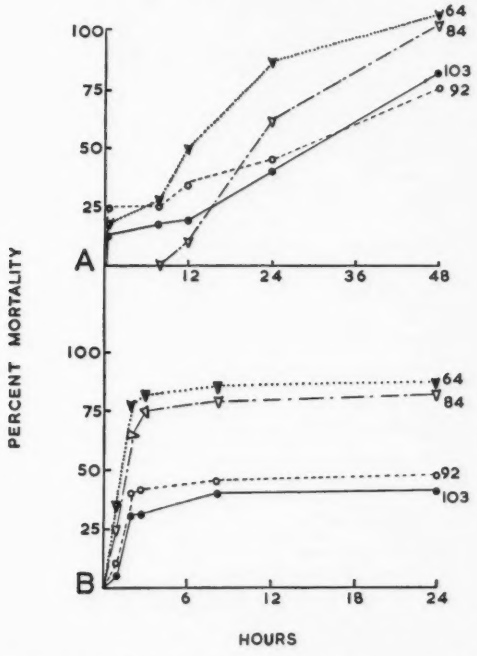



FIG. 3. Temperature tolerance of goldfish fed hydrogenated pilchard oil. Values on graph are iodine numbers of oils extracted from fish. *A*, low temperature test; *B*, high temperature test.

*High Temperature Tolerance*

At the test temperature of 35.5° C. the times for 50% mortality of fish fed pilchard oil, herring oil, and lard were 50, 45, and 85 min. respectively. These values are comparable with 33.5, 35, and 60 min. reported earlier for fish treated with the same three fats and tested at 36.0° C. (6). The present data confirm the conclusion (6) that the lard diet changed the heat tolerance significantly from that of fish fed on low fat diets or on diets rich in pilchard oil or herring oil.

When goldfish have been fed hydrogenated pilchard oils the order for death at high temperature is the same as that which occurs at low temperatures. Hydrogenation of the pilchard oil used in the diet reduces the resistance to both low and high temperatures (Figs. 3 and 4). The differences were particularly marked in the first series (Fig. 3) but the order is the same in each case.

Although the fish fed cottonseed oil and "Crisco" did not respond well to the diet, the mortality curves follow the same order as those of the fish that

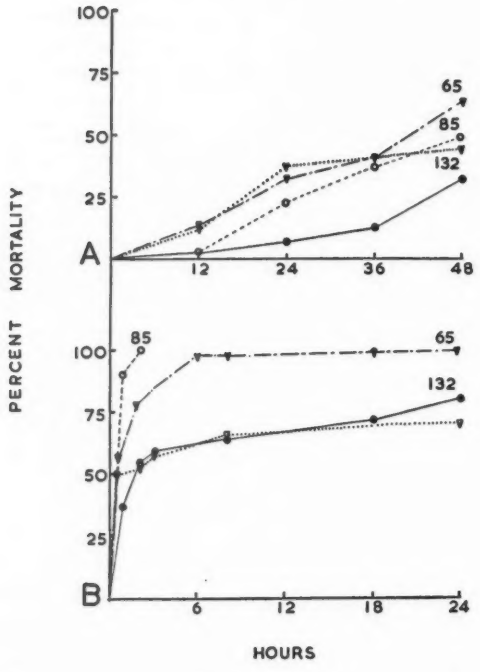



FIG. 4. Temperature tolerance of goldfish fed hydrogenated pilchard oils and lard (dotted line). Values on the graphs are iodine numbers of the oils extracted from fish. *A*, low temperature test; *B*, high temperature test.

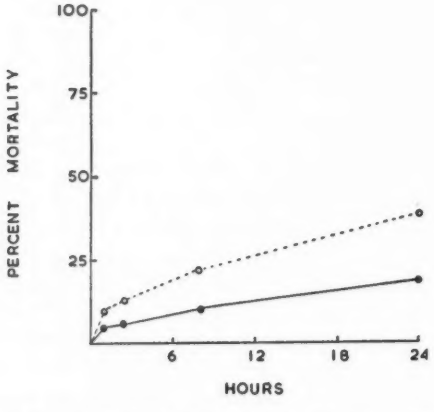



FIG. 5. Temperature tolerance of goldfish fed on cottonseed oil, continuous line; and "Crisco", broken line. Test temperatures 33.9 - 35.8° C.

were fed pilchard oil. It is evident that the resistance of goldfish to high temperature is likewise lowered by hydrogenation of cottonseed oil used in preparation of the diet (Fig. 5).

## Discussion

The lipids of the body are divided into two groups, the depot fats or fuel reserves ('variable element') and the structural fats which are a constant part of all protoplasm ('constant element'). It is the latter which enter into the composition of the cell membranes and are evidently related to thermal tolerance in poikilotherms.

Ingested fat accumulates readily in the fat depots but does not become part of the fabric of the organism so quickly (1). It has been shown, however, that under certain conditions ingested fats are metabolized by the liver (1) and may eventually be found in organs such as liver, intestinal mucosa, and blood (7). The lipids of nerve and muscle are modified much more slowly (7).

No information is available on the time required to modify the "constant element" of goldfish nor the degree of change which may be possible, but a comparison of the two series of fish fed pilchard oil suggests that the change may be gradual (Table I, Figs 3 and 4). The goldfish in the first series showed a greater range in thermal resistance than those in the second series. The first series was fed for 60 to 65 days; the second only 50 days.

The experiments were designed to test the theory that thermal resistance is in some way related to the melting point of the fats. Such a simple theory is not tenable unless it is assumed that the dietary fat does not modify the protoplasmic lipid. Fish fed on the hardest natural fat (lard) were most resistant to heat. Resistance to cold showed no relationship whatever to the melting points of natural fats. Fish fed on the hardest form of hydrogenated pilchard oil were least resistant to both heat and cold. The postulated relationship to melting point is not, therefore, evident. It seems that some factor other than melting point of fat must be responsible for the difference in survival time of these fish.

Several possible explanations may be advanced for the increased temperature susceptibility shown by goldfish feeding on hydrogenated pilchard oil. Some essential dietary fatty acid might be destroyed or changed by hydrogenation. Since, however, the basic diet of "Pablum" maintains goldfish in evident good health for long periods and since essential fatty acids are required in only small amounts, this explanation does not seem likely. Again, it is possible that the hydrogenated pilchard oils are less readily available in the metabolism of the fish and that the earlier death of fish feeding on them is due to an added metabolic stress. Oxygen consumption, measured under resting conditions by the method of Fry and Hart (4), was essentially the same in the different groups and did not suggest that hydrogenated fat added an extra metabolic load. In fact, the oxygen consumption was slightly lower for fish feeding on the hydrogenated oils (unpublished data).

The relationship between thermal resistance and degree of unsaturation in the series of hydrogenated oils suggests that the presence of double bonds may be important. If temperature tolerance depends on the integrity of a plasma membrane made up of a protein–lipid–calcium complex (5) it is possible that the unsaturated fats are, in some way, more effective binders by virtue of the double bonds. The integrity of the plasma membrane may depend on oxidative processes or other electron transfers where the unsaturated molecules act as hydrogen acceptors. Stimulating effects of extreme temperature may exhaust this mechanism and result in loss of calcium to the interior of the cell as postulated by Heilbrunn (5) or change permeability in some other way. Studies in progress show that differences in water content of tissues develop during temperature acclimatization and suggest that an unfavorable water balance is one factor involved in temperature death.

## Acknowledgments

B. E. Bailey of the Pacific Fisheries Experimental Station, Fisheries Research Board of Canada, has provided ready advice and stimulating suggestions on many occasions. Lyle Swain of the same Station, made the experiments possible by hydrogenating the pilchard oil. Neal Carter, Director of the Station, has welcomed and encouraged our visits. W. Chalmers of Western Chemicals, Ltd., provided the fish oils for the study. It is a pleasure to acknowledge the assistance so cheerfully given.

## References

1. BALDWIN, E. Dynamic aspects of biochemistry. Cambridge University Press. 1948.
2. BROWN, J. B. The nature of the highly unsaturated fatty acids stored in the lard from pigs fed on menhaden oil. J. Biol. Chem. 90 : 133-139. 1931.
3. ELLIS, N. R. and ISBELL, H. S. Soft pork studies. II. The influence of the character of the ration upon the composition of the body fat of hogs. J. Biol. Chem. 69 : 219-238. 1926.
4. FRY, F. E. J. and HART, J. S. The relation of temperature to oxygen consumption in the goldfish. Biol. Bull. 94 : 66-77. 1948.
5. HEILBRUNN, L. V. An outline of general physiology. 2nd ed. W. B. Saunders Company, Philadelphia. 1943.
6. HOAR, W. S. and DORCHESTER, J. E. C. The effect of dietary fat on the heat tolerance of goldfish (*Carassius auratus*). Can. J. Research, D, 27 : 85-91. 1949.
7. SINCLAIR, R. G. The metabolism of the phospholipids. VIII. The passage of elaidic acid into tissue phospholipids. Evidence of the intermediary rôle of liver phospholipid in fat metabolism. J. Biol. Chem. 111 : 515-526. 1935.

# SOME EFFECTS OF TEMPERATURE ACCLIMATIZATION ON THE CHEMICAL CONSTITUTION OF GOLDFISH TISSUES[1]

By William S. Hoar[2] and Merva K. Cottle[3]

## Abstract

The water content of goldfish maintained at 5°, 15°, 20°, and 35° C. varied directly and the lipid content varied inversely with the change in acclimatization temperature. The cholesterol: fatty acid ratio varied directly and the cholesterol : phospholipid ratio varied inversely with the water content and the temperature of acclimatization. The iodine value changed by 0.51 point per centigrade degree increase in acclimatization temperature These changes are discussed in relation to thermal tolerance and temperature death in poikilotherms.

## Introduction

Several variations in chemical constitution of tissues have been associated with differences in environmental temperature. In plants and poikilotherms it has been noted that individuals maintained at lower temperatures have lower melting point fats than the members of the same species living at higher temperatures (9). The water content of tissues may also vary with the environmental temperature (9). It is probable that such biochemical differences are in some way responsible for the change in temperature tolerance, but the significance of the various changes is still theoretical. A careful analysis of the biochemistry of temperature acclimatization should lead to an understanding not only of the part played by these different constituents in acclimatization but also the nature of thermal death of tissues. In the work described here, an attempt has been made to follow changes in water content and in the content of several lipid fractions during temperature acclimatization of goldfish and to examine the results in relation to temperature death.

## Materials and Methods

Three experiments, referred to as *A*, *B*, and *C*, were carried out. Numbers of goldfish (*Carassius auratus*) and acclimatization temperature data are given in Table I. In general, temperatures were raised or lowered 1° C. per day until the desired temperature was reached. Fish were held for 10 days or more at that temperature before analyses of tissues were carried out. Goldfish were retained in lots of 100 (50 in Experiment *A*) in thermostatically controlled aquaria. The water was changed once or twice a week. Each lot of fish was fed 15 to 20 gm. "Pablum" (Mead-Johnson and Co.) three times per day. Once per week this diet was supplemented by 25 gm. of dog meal (Gaines).

[1] *Manuscript received September 27, 1951.*

*Contribution from the Department of Zoology, University of British Columbia, Vancouver, B.C., with financial assistance from the National Research Council of Canada.*

[2] *Professor of Zoology and Fisheries.*

[3] *Graduate Student.*

Temperature tolerance was determined only for fish of Experiment *B*. For this a large tank divided into three areas by plastic netting provided identical temperature conditions for the three groups. The time required to kill 90% to 100% of the sample at the particular temperature is given in Table I.

In Experiment *A* moisture content was determined by drying individual fish to constant weight in test tubes at 95° to 100° C. (six fish dried at 15° and eight fish at 35°). All other analyses were carried out by sampling tissue from lots of four to six goldfish minced in a Waring Blendor. Determinations were made in triplicate and any discrepancies carefully checked.

In general, moisture was determined by drying 20- to 30-gm. samples to constant weight in an oven at 95° to 100° C.; total lipid was measured by shaking a definite weight of minced tissue with cold peroxide-free ether and anhydrous sodium sulphate and repeating the extraction with several successive portions of fresh ether. The extracts were combined, the ether distilled off *in vacuo* at 38° C., the oil dried in a vacuum desiccator and weighed.

Oil, extracted as described above, was analyzed by Bloor's methods for phospholipid (4) and cholesterol (3, 5). Values reported for fatty acids are differences between total lipid and cholesterol. The iodine number was determined by the routine Wijs' method. We are indebted to B. E. Bailey, Pacific Fisheries Experimental Station of the Fisheries Research Board of Canada, and C. G. Wilber, Biological Laboratories, St. Louis University, for advice on the various techniques.

## Results

The data are summarized in Table I and presented graphically in Fig. 1. It is evident from Table I that the acclimatization period was long enough

TABLE I

ACCLIMATIZATION DATA AND MORTALITY TIMES FOR GOLDFISH USED IN EXPERIMENTS. LOTS OF 50 FISH AT EACH TEMPERATURE IN EXPERIMENT *A* AND 100 AT EACH TEMPERATURE IN EXPERIMENTS *B* AND *C*

| Experiment | Starting date | Acclimatization temperature | | | Mortality time, 90-100% | |
|---|---|---|---|---|---|---|
| | | Final, ° C. | Days required to change temperature | Days at acclimatization temperature | 1.9-2.3° C. | 35.9-36.2° C. |
| *A* | July 8/49 | 15 | 0 | 33 | — | — |
| | | 35 | 20 | 12 | — | — |
| *B* | May 15/50 | 5 | 0 | 25 | Survived | 15 min. |
| | | 20 | 16 | 9 | 18 hr. | 7 hr. |
| | | 35 | 15 | 12 | 35 min. | Survived |
| *C* | July 25/50 | 5 | 15 | 12 | — | — |
| | | 20 | 0 | 22 | — | — |
| | | 35 | 16 | 12 | — | — |

to affect temperature tolerance in the goldfish of Experiment *B*. Times for the other groups were comparable and it is reasonable to conclude that acclimation had taken place.

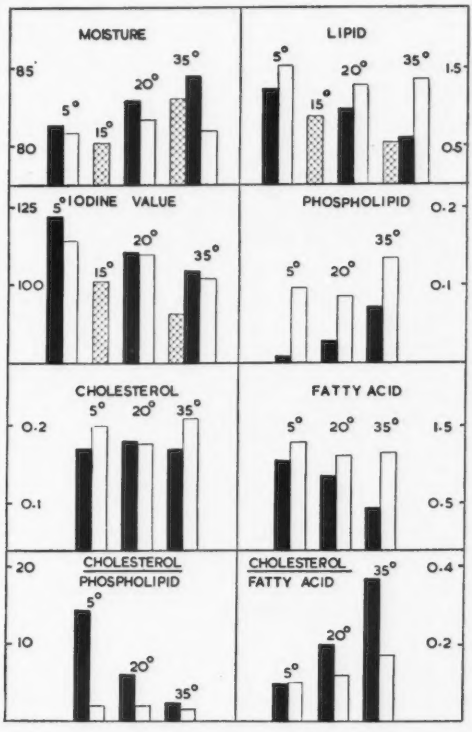



FIG. 1. Changes in chemical constitution of tissues from goldfish acclimatized to different temperatures. Values expressed in per cent of wet tissue. Cross-hatched bars, Experiment *A*; black bars, Experiment *B*; open bars, Experiment *C*.

Data for the chemical analyses, presented graphically in Fig. 1, show consistent changes in iodine values, in the ratios of cholesterol to phospholipid, in the ratios of cholesterol to fatty acid and, with one exception, in the moisture and lipid content. This one exception is for the group of fish acclimatized to 35° C. in Experiment *C*. No explanation is offered. It is probable, however, that this value is not truly representative.

Fig. 1 shows that the changes were more consistent and generally more marked for fish of Experiments *A* and *B* than for those of Experiment *C*. Acclimatization times were comparable (Table I). The fish were, however, procured at different seasons. Stage of sexual maturity or some other physiological condition may have been responsible for the variation.

## Discussion

The decrease in lipid content with the rise in temperature is probably due to an elevation in rate of metabolism. Increased oxygen consumption for goldfish maintained at higher temperatures has been demonstrated (7, 8). With the greater utilization of food, less fat is stored in the depots and the total body fat decreases.

Increased tissue water in goldfish maintained at high temperatures was also observed by Black (2). In her experiments the water content of fish acclimatized to 2°-11° C. was 80% and of fish held at 11°-26° C. was 82% (mean values for 8 and 13 fish respectively).

Two factors may be responsible for the increased tissue water at higher temperatures. In the first place, increased oxidation of fat produces more metabolic water (1). In the second place, water enters cells much more rapidly at higher than at lower temperatures (9). This latter effect will be particularly important in an animal such as the goldfish, which is hypertonic to its environment and it seems likely that the increased metabolic water will have an insignificant effect in comparison with the increased rate of diffusion and permeability. Loeb and Wasteneys (13) found that *Fundulus* tolerated maximum temperatures in hypertonic solutions. They postulated that a rise in temperature brought about changes in permeability of the surface body cells which resulted in death. In experiments on *Fundulus* in sea water Doudoroff (6) found that controls at 14° C. contained more water than those at 20° C. In this case, the hypotonic fish lost water possibly owing to increased permeability at higher temperatures. In the hypertonic goldfish the flow of water will be in the opposite direction and, as reported here, the fish accumulate water at higher temperatures.

Changes in the ratio of cholesterol to phospholipid and in the ratio of cholesterol to fatty acids during acclimatization may, to some extent, control this accumulation of water. Mayer and Schaeffer (15) found the ratios of cholesterol to fatty acid and cholesterol to phospholipid to vary directly as the water content of the tissues. In the present study the water content varied directly as the cholesterol/fatty acid ratio but inversely as the cholesterol/phospholipid ratio. Wilber and Del Pomo (16) reported similar differences in ratios between Arctic and non-Arctic fish. The discrepancy between these results and those of Mayer and Schaeffer (15) may be due to variation in different groups of animals and organs. The essential point is the evident relationship between water permeability, water balance, and the nature of the protoplasmic lipids (14).

Finally, temperature acclimatization modified the degree of unsaturation of the body fats. Hunter (12) reported an average change of 0.5 points in iodine value per degree change in acclimatization temperature of goldfish. The increase recorded in the present study is 0.51 points. It has been suggested that heat death is related to the melting point of the protoplasmic fats (9). According to this view the essential feature of temperature death is the disorganization of the protein–lipid–calcium complex which forms the

plasma membrane. Organisms with more solid fats tend to withstand higher temperatures since the fats do not melt so readily and destroy the integrity of the membrane. A feature of acclimation to high temperature is thus seen to be the elevation of lipid melting point. A different explanation for this change in unsaturation of fats with temperature is suggested by a study of the effects of dietary fats on temperature tolerance.

When goldfish are fed different types of fat in high concentration their thermal resistance is altered (10, 11). A series of diets containing hydrogenated pilchard oils produced a lowered resistance to both low and high temperatures with hydrogenation (10). It was suggested that the unsaturated double bonds are important in the maintenance of the permeability of the plasma membrane. If this is the case, the increase in iodine value observed at lower temperatures will be of importance in controlling permeability. It seems reasonable to assume that the integrity of the plasma membrane and its permeability will be dependent upon active metabolism of an oxidative nature. At lower temperatures when the rate of metabolism is depressed the additional double bonds in the more unsaturated lipids may serve as additional hydrogen acceptors or may otherwise promote metabolism there.

Thus, the findings indicate that temperature acclimatization changes the permeability properties of cell membranes. At higher temperature the change in the *coefficient lipocytique* (14) compensates for the increased permeability by controlling water imbibition or passage. At lower temperatures the development of more higher unsaturated lipids promotes phases of metabolism involving double bonds and maintaining the integrity of the membrane.

## References


1. BALDWIN, E. Dynamic aspects of biochemistry. Cambridge University Press. 1948.
2. BLACK, V. S. Personal communication. 1950.
3. BLOOR, W. R. The determination of cholesterol in blood J. Biol. Chem. 24 : 227-231. 1916.
4. BLOOR, W. R. The oxidative determination of phospholipid (lecithin and cephalin) in blood and tissues. J. Biol. Chem. 82 : 273-286. 1929.
5. BLOOR, W. R. The cholesterol content of muscle. J. Biol. Chem. 114 : 639-648. 1936.
6. DOUDOROFF, P. The resistance and acclimatization of marine fishes to temperature changes. II. Experiments with *Fundulus* and *Atherinops*. Biol. Bull. 88 : 194-206. 1945.
7. FREEMAN, J. A. Oxygen consumption, brain metabolism, and respiratory movements of goldfish during temperature acclimatization, with special reference toward lowered temperatures. Biol. Bull. 99 : 416-424. 1950.
8. FRY, F. E. J. and HART, J. S. The relation of temperature to oxygen consumption in the goldfish. Biol. Bull. 94 : 66-77. 1948.
9. HEILBRUNN, L. V. An outline of general physiology. 2nd ed. W. B. Saunders Company, Philadelphia. 1943.
10. HOAR, W. S. and COTTLE, M. K. Dietary fat and temperature tolerance of goldfish. Can. J. Zool. 30 : 41-48. 1952.
11. HOAR, W. S. and DORCHESTER, J. E. C. The effect of dietary fat on the heat tolerance of goldfish (*Carassius auratus*). Can. J. Research, D, 27 : 85-91. 1949.
12. HUNTER, J. G. The change in degree of unsaturation of body fats during acclimation of goldfish (*Carrassius auratus*) to high temperature. M.A. thesis, University of British Columbia. (Unpub.) 1948.

13. LOEB, J. and WASTENEYS, H. On adaptation of fish (*Fundulus*) to higher temperatures. J. Exptl. Zool. 12 : 543-557. 1912.

14. MAYER, A. et SCHAEFFER, G. Recherches sur les constantes cellulaires teneur des cellules en eau. I. Discussion theorique. L'eau, constante cellulaire. J. physiol. et path. gén. 16 : 1-16. 1914.

15. MAYER, A. et SCHAEFFER, G. Recherches sur les constantes cellulaires teneur des cellules en eau. II. Rapport entre la teneur des cellules en lipoides en leur teneur en eau. J. physiol. et path. gén. 16 : 23-38. 1914.

16. WILBER, C. G. and DEL POMO, M. Comparative study of lipids in whole carcasses of Arctic and non-Arctic fish. Proc. Soc. Exptl. Biol. Med. 72 : 418-420. 1949.

# SOME ASPECTS OF MOISTURE ABSORPTION AND LOSS IN EGGS OF *MELANOPLUS BIVITTATUS* (SAY)[1]

By R. W. Salt[2]


## Abstract

The penetration of moisture into or out of eggs of *Melanoplus bivittatus* (Say) is assisted or retarded by a variety of envelopes. Proceeding inwardly these are, in the newly laid egg, the chorion, the primary lipoid layer (probably waxy), and the vitelline membrane; later, to these are added the secondary lipoid layer (probably oily), the yellow cuticle, a bonding material, the white cuticle, and the serosa, which secretes all the others of this group. The primary lipoid layer waterproofs the egg in its earliest stages, but its function is soon taken over by the secondary lipoid layer. A specialized moisture-permeable area, the hydropyle, is formed at the posterior pole. Moisture is absorbed from the environment chiefly through the hydropyle, reaching a peak of absorption on the eighth or ninth day at 25° C. and then decreasing because of the limited expansion of the cuticular layers. The original moisture content of the egg is approximately doubled. Moisture loss in young eggs, up to seven days of age, is restricted at first by the primary lipoid layer and later by the secondary lipoid layer. From the eighth day to the end of anatrepsis, on the 14th day, the desiccation rate is greatly increased. Most of the loss occurs through the hydropyle, which at this time is normally absorbing water; however, the relationship between loss and gain through the hydropyle is not close. After blastokinesis, or after the 14th day of incubation at 25° C., the desiccation rate is extremely variable. Some of the moisture loss takes place through the hydropyle, and when the rate is low all of it does so; however, in many eggs the general cuticular surface becomes permeable (to desiccation but not to absorption), and the losses are erratic and unpredictable. With the gradual onset of diapause after the 21st day, the desiccation rate declines. Changing the moisture conditions during incubation of the eggs results in changes in the desiccation rates. The variability of the rates in older eggs was not caused by retardation of development, irregular moisture absorption, intermittent drying, or laboratory techniques, but was probably the result of variable cuticular permeability. Most *M. bivittatus* eggs, regardless of age, survived losses up to one-third of their moisture content, but few survived more than a two-thirds loss. After more than a one-third loss, the collapsing action of the egg frequently produced distorted or abnormal embryos. Though these often lived for some time, they never hatched. Those individuals and those age groups with the lowest desiccation rates naturally survived dry conditions longest.


## Introduction

The problem of water conservation by insects has of late received considerable attention. Its main feature is the waterproofing of the cuticle with lipoid materials, either in layers or by impregnation. The problem in acridid eggs is marked by certain additional facts that render it less simple. The original chorionic waterproofing breaks down and is replaced with cuticular waterproofing. At the posterior pole of the egg, however, the cuticle is permeable, allowing for the absorption of moisture that is essential to the later development of the embryo. Under dry conditions water is lost through this specialized area. The egg is thus faced with the necessity of absorbing

[1] *Manuscript received September 26, 1951.*

*Contribution No. 2879, Division of Entomology, Science Service, Department of Agriculture, Ottawa, Canada.*

[2] *Senior Entomologist, Field Crop Insect Laboratory, Lethbridge, Alta.*

water from a moist environment while at the same time protecting itself against the desiccating action of a dry one.

In these investigations the principal emphasis has been placed on the quantitative aspects—the gains and losses by eggs under various controlled conditions. Such a study, however, requires some knowledge of the egg envelopes, for these are the barriers that by their physical and chemical nature protect the egg contents against both dryness and excessive moisture. This was obtained by observation of fresh material in gross aspect and by chemical tests on it, rather than by the usual methods of fixing and sectioning. The works of Jahn (3), Slifer (7-11), and Shutts (6) on eggs of *Melanoplus differentialis* (Thomas) were of great help because of the similarity of material. However, many differences in structure and function were found.

## The Layers Covering the Egg

### The Chorion

The chorion is a tough covering that protects the egg and gives it shape during the first part of its development. It is secreted by the follicular cells of the mother and bears their imprint on its outer surface. Like that of *M. differentialis* eggs, described by Jahn (3) and Slifer (7), it is composed of an outer, fairly dense layer, and an inner, granular, absorbent layer having a glazed inner surface appressed to the vitelline membrane. The outer layer is somewhat water-repellent, but since water enters readily at the micropylar ring, quickly penetrating the spongy inner layer, the outer waterproofing is not very effective. The smooth inner surface of the chorion appears to be waterproofed by a lipoid coating like that demonstrated by Slifer (11) in *M. differentialis* eggs and corresponding to Beament's (1) "primary wax layer". The waterproofing is not perfect, however, for small losses are registered in unsaturated air and gains are detectable after four or five days of incubation on contact water after oviposition. Its effectiveness is therefore limited to the earliest stages in the development of the egg.

### The Vitelline Membrane

The vitelline membrane is a thin, transparent, noncellular membrane lying between the chorion and the yolk. If a newly laid egg is placed in sodium hypochlorite the chorion disintegrates, leaving the egg encased in the vitelline membrane. It is sufficiently tough to retain the original shape of the egg when submerged but ruptures very easily when handled with forceps. The thinnest and weakest portion is the posterior tip, behind the micropylar ring, and this is also the most permeable portion. Sodium hypochlorite can be seen to attack the yolk at the posterior tip of the egg shortly after the last vestiges of chorion have disappeared.

During the first three or four days of incubation at 25° C. the vitelline membrane is the only layer discernible beneath the chorion. It gradually becomes underlaid by the serosa and later by the two layers of cuticle formed

by the serosa. By the seventh day the egg has expanded somewhat, having absorbed water to the extent of about 11% of its weight. During the next few days the vitelline membrane will be found to be ruptured if the chorion is removed in sodium hypochlorite, but it adheres closely to the cuticle unless rubbed off. By the 11th or 12th day the pieces of vitelline membrane peel off the cuticle rather easily in sodium hypochlorite, and more readily yet if then transferred to water. However, after the cuticle is formed, beginning on the sixth day, the vitelline membrane appears to be attached to it at the micropyles that form a ring around the egg a short distance in front of the posterior pole. The significance of this is obscure. The spermatozoa apparently must penetrate the vitelline membrane or else pass through openings underlying the micropylar tubes in the chorion. In either case the need for attachment to a cuticular layer formed six days after fertilization is not clear.

It has not been found possible to examine the vitelline membrane in young eggs without the aid of sodium hypochlorite, which removes the chorion and may also affect the vitelline membrane. The membrane peels off the cuticle of older eggs with ease, however, and has the same appearance as when treated with sodium hypochlorite. Its role in conserving water is therefore problematical. It may interfere with the transfer of water into or out of the egg, except at the posterior tip, until the swelling of the egg ruptures it. From then on, it adheres more loosely to the cuticle and cannot play much part in waterproofing.

## The Serosa and the Hydropyle Cells

The serosal membrane is formed gradually by the multiplication of a few cells that gather at the surface of the yolk. By appropriate technique it can be demonstrated as a discrete membrane as early as the third day of incubation at 25° C., though its beginning as a few multiplying cells precedes this. Growth proceeds until on the sixth day the cells are closely packed and hexagonal, approximating 0.11 mm. diagonally, with large, spherical nuclei located centrally. By this time the serosa is completely formed and it begins to function. It secretes two cuticular layers and a waterproofing layer, and provides a specialized water-absorbing area, the hydropyle, regulated by modified serosal cells.

At the posterior pole the serosal cells are somewhat thicker than elsewhere and the hexagonal shape is less apparent. Underlying as they do that area of the egg envelopes which is especially adapted for water transfer, they are in a position to control or at least influence the intake of water or other liquids. Slifer (9) named them the hydropyle cells; together with the specialized membranous areas covering them they form the hydropyle. Recently Slifer (12) showed that in *M. differentialis* eggs the hydropyle cells secrete a waxy substance that apparently permeates or covers the outer cuticle at the posterior pole and protects the egg against excessive water loss during diapause. In *M. bivittatus*, diapause occurs at a much later embryonic stage and is not

obligate. More often than not in Western Canada the eggs overwinter at an earlier stage and never enter diapause. It will be seen later that the hydropyle cells of *M. bivittatus* can scarcely provide waterproofing during diapause as they do in *M. differentialis*, because by this time the entire cuticular surface may be permeable.

On the 14th day the serosa, appearing much as it did when first fully formed, pulls away from the cuticle near the posterior pole. The hydropyle cells remain attached to the cuticle, so that a serosal cylinder or sleeve is formed between the hydropyle and the head of the embryo. A transparent fluid fills the space inside and outside the constricted zone. The sleeve gradually decreases in diameter until it becomes a mere thread, which then breaks, leaving the embryo and yolk encased in a shortened serosal membrane. This takes place on the 14th day; during the next 24 hr. the embryo begins blastokinesis, breaking through the serosa in the process. By the time that revolution is complete, on the 16th day, the open edge of the serosal bag has moved anteriorly, constricting the yolk immediately in front of the embryo. During the next three days the embryo grows forward and the yolk, with its covering serosa, is gradually engulfed. By the end of the 18th day dorsal closure is complete.

## The Cuticular Layers

The serosa secretes two cuticular layers that are similar if not identical to those described by Jahn (3) for *M. differentialis*. The outer or yellow cuticle is a thin, apparently homogeneous membrane, whereas the inner or white cuticle is tough and fibrous in texture. Almost as soon as the serosa becomes fully formed, on the sixth day, the outer cuticle is present in its final form, indicating that its deposition or formation is very rapid. The serosa also starts to lay down the white cuticle on the sixth day, adding to its thickness for several days. By the 11th day the white cuticle appears to be fully formed; it has a translucent, milky-white coloration, bears a faintly imprinted pattern corresponding to the serosal cells, and when torn exhibits a fibrous texture.

The yellow cuticle is waterproofed on its outer surface by the secondary lipoid layer, probably oily in nature. It also is secreted by the serosa, and must be laid down before the yellow cuticle.

Separation of the yellow and white layers by mechanical means was not found possible in *M. bivittatus* eggs. However, if a piece of cuticle is placed in sodium hypochlorite the layers soon separate and the yellow cuticle rolls off the white. In large segments, such as a cuticle complete except for one end, the yellow cuticle can be pulled off as a limp bag. It tears readily, in contrast with the remaining white cuticle, which holds its shape. Under a dissecting microscope the yellow cuticle appears to be homogeneous, even at the posterior pole. The white cuticle, on the other hand, is distinctly different in this area, being thinner and transparent. Here it is not fibrous and it tears like the yellow cuticle and the vitelline membrane, with a clean shear.

The yellow cuticle does not separate from the white in sodium hypochlorite if the egg is intact; even after an hour, if the egg is then transferred to water

and dissected, the two layers do not separate. The readiness with which the yellow cuticle peels off the white in a large segment indicates that the action is not confined to a torn edge, but that it takes place through the white cuticle.

## Water Transfer

Eggs of *M. bivittatus*, like other acridid eggs, must absorb water from their environment in order to complete their development. Under dry conditions they lose moisture with more or less ease. Obviously losses are affected by previous gains, and vice versa. However, the relationship is neither simple nor direct. Water is gained as free water but is rapidly incorporated into the various components of the egg. On the other hand, water is normally lost only by evaporation from the surface of the egg; the tissues supply it through a gradient depending not only on proximity to the site of loss but also on forces such as those of adsorption and osmosis. Physical barriers, such as hydrophobic layers, also affect the transfer. The development of the embryo and the concurrent changes in the rest of the egg are affected by water supply; for example, blastokinesis cannot begin until the water content has been raised to a certain level.

### Normal Moisture Absorption

This subject has been partly covered in a previous paper (5). The average egg, supplied with free water at 25° C., absorbs very small amounts during the first few days, but the rate increases detectably. At the end of five days only 2% has been added to the weight of the egg. This is the period before the serosa and cuticular layers appear. The rate then increases rapidly, reaching a peak on the eighth and ninth days, by which time 40% has been added to the original weight of the egg, and the various egg coverings, now including the cuticular layers, are becoming greatly stretched. By this time the forces pulling water into the egg are acting against the elasticity of the egg coverings and of necessity the rate of uptake drops rapidly. A second, minor increase in rate and subsequent decrease, noted in many eggs (5), is not consistent and has not been explained. The total absorption normally amounts to about 60% of the oviposition weight, or in other words the original moisture content is approximately doubled. About 88% of this is absorbed during anatrepsis, before blastokinesis, for which process it is essential.

Eggs that are denied access to free water but are protected from excessive drying develop to the end of anatrepsis. Here they enter a sort of diapause that is terminated when free water is supplied; water is absorbed rapidly, nearly 50% in two days, and within three days blastokinesis begins.

Between these extremes of continuous free water and none at all, eggs absorb water when it is present and lose it when dry. Their moisture content must be raised to a certain level, however, before blastokinesis can begin. In a dry environment an egg is theoretically safer to be under that level than to be barely over it.

When the egg is laid it needs protection against desiccation but because of its later need to absorb water, the waterproofing mechanism must be adjustable. The primary lipoid layer underlying the chorion, aided by the semi-hydrophobic character of the outer surface of the chorion and possibly by the vitelline membrane, assures a fair degree of waterproofing. It is not perfect, however, for about 2% is consistently gained by newly laid eggs incubated for five days on free water. Water readily penetrates the chorion through the micropyles, soaking the spongy endochorion; in addition, the posterior pole of the vitelline membrane is permeable to water. The main barrier is therefore the primary lipoid layer, which must be imperfect. This is indicated not only by the small gain observed during this period but also by the fact that eggs of this age lose small amounts of moisture in unsaturated air. The breakdown of the primary lipoid layer can hardly be attributed to the action of microorganisms or to aging (suggested by Slifer (10) as possible reasons for the disruption of the waxy layer over or in the hydropyle of *M. differentialis* eggs in diapause), because the timing is too consistent. The possibility that minute cracks develop in the layer and allow some absorption, which in turn swells the egg and aggravates the cracking, is unlikely, at least as a complete explanation. If such were the case the handling of very young eggs in laboratory experiments would disrupt the layer and produce erratic results, whereas actually this age group gave the most consistent results for both absorption and loss. It will be shown later that the increase in absorption rate begins on about the fourth day, whereas the increase in desiccation rate does not take place until the eighth day at least.

On the sixth day the hydropyle cells are present and presumably functioning; so is the yellow cuticle, which is almost impermeable to water except at the hydropyle. Therefore, though water is probably absorbed principally at the posterior pole of the egg before the sixth day, it is certainly absorbed there for some time thereafter. Sealing the hydropyle stops the absorption, showing that the hydropyle is the site of entry (5, 9). However, this does not tell us much about the method of entry—whether absorptive, adsorptive, or capillary; whether osmotic, diffusive, or otherwise; whether operated and controlled by the hydropyle cells, the overlying cuticular layers, or a combination of the two. To investigate this aspect of the problem, eggs of *M. bivittatus* were allowed to absorb water at different temperatures. The eggs used in this experiment had been kept at 25° C. and 90% relative humidity from the day of oviposition until three to six weeks old. Under these conditions anatrepsis is completed in somewhat less than three weeks, and the eggs are highly receptive to free water. Fig. 1 shows the course of absorption for 10 days at 0°, 5°, 10°, and 25° C. To ensure fluidity at 0° C. a 0.1 *N* sodium chloride solution was used at all temperatures. A second set of eggs at 25° C., supplied with distilled water, showed no significant differences from those supplied with the dilute salt solution.

The uptake of water is markedly affected by temperature. If the action were simply osmotic, the rates would be proportional to absolute temperature;

if chemical, the rates would double or triple for a 10° C. rise in temperature. For the straight parts* of the curves in Fig. 1 the $Q_{10}$ is roughly 10-12. Absorption by grasshopper eggs is therefore accomplished by a force greater than

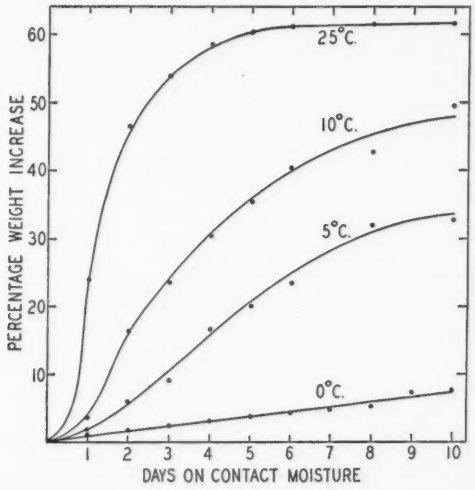



FIG. 1. Absorption of moisture by "dry-reared" eggs of *M. bivittatus* at 0°, 5°, 10°, and 25° C.

that of osmosis, though osmosis is a contributing factor. Absorption by chemical reaction plus osmosis is scarcely sufficient to fit the observations. Additional factors such as electro-osmosis and imbibition may supplement the action. It was thought possible that the hydropyle cells might perform the absorption as an active, vital transfer. However, when dead partially dehydrated eggs were supplied with free water many of them absorbed amounts as great as did living eggs. But other eggs comparable to those of Fig. 1, except that they were frozen first, absorbed only small amounts of water. From such variable results it appears that the living condition was not invariably necessary for rather large amounts of absorption.

It is difficult to be sure that the egg cuticle is perfectly intact, and this fact alone renders quantitative data on water uptake open to suspicion. After the cuticle is well formed it is a fairly simple matter to peel off the chorion with a pair of forceps. In most cases, provided the egg is turgid, even a slight scratching of the cuticle with forceps raises a tiny droplet of liquid to the surface. This droplet must have rapidly penetrated both inner and outer cuticle, and perhaps the serosa also. It does not, however, serve as a release for sufficient liquid to relieve the turgidity of the egg, for the amount remains small and further contact produces similar droplets. The liquid dries fairly rapidly, but not sufficiently so for this to be the cause of stoppage; in fact,

** Since absorption acts against the elasticity of the egg coverings, only the more or less straight parts of the curves are readily comparable.*

if a small area is scratched, rather than a point or a line, the liquid will cover the whole area and may remain fluid for considerable time. Even under such conditions the turgidity is not noticeably reduced. It therefore seems likely that the path of egress through the membranes is blocked within the membranes and not within the exuded liquid. Eggs vary greatly in this reaction; some have to be scraped with a sharp object before they will "bleed", whereas others will respond to a light touch with a smooth object. Experiments designed to test the water loss from such eggs failed to reveal a correlation between water loss and "bleeding"; nor did eggs lose appreciably more water after being extensively scratched than they did before.

Slifer (9) and Salt (5) showed that water is absorbed principally through the hydropyle. This does not rule out the possibility of some water interchange through the general cuticular surface. As will be shown later, water may be lost through the cuticle, and one would expect some absorption through it.

To test this point, eggs were allowed to dry until they had lost their turgidity and started to collapse. In this condition the chorion may be readily chipped off the posterior pole, leaving the hydropyle and the entire micropylar ring area exposed. No "bleeding" resulted from this treatment. The hydropyles were then covered with liquid solder as a precautionary measure, and after weighing, the eggs were placed, hydropylar ends up, in shallow holes made in wet blotting paper. The chorions conducted moisture upwards as far as their torn edges, thus keeping the enclosed cuticles wet except at the posterior tips. The dishes of eggs were kept at 25° C. and 95% R. H. to prevent excessive drying at the exposed tip. The liquid solder also protected against drying, as well as against absorption of chance condensation moisture. Control eggs, with the chorion removed from the posterior tips, but not soldered, and with the hydropyles down in the wet blotter, regained their turgidity in a day or two. Four sets of experimental eggs made only very slight gains in periods up to eight days, as shown in Table I. Many of these eggs did not register

TABLE I

PERCENTAGE WEIGHT GAINS MADE BY *M. bivittatus* EGGS WHEN ABSORPTION THROUGH THE HYDROPYLE WAS ELIMINATED

| Age, days | No. of eggs | Days on contact moisture | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 14 | 12 | — | 0.2 | — | 1.5 | — | — | — | 3.4 |
| 14 | 17 | — | 1.2 | — | 1.2 | — | — | 3.2 | — |
| 17-20 | 16 | 0.1 | — | — | — | — | 3.0 | — | — |
| 17-20 | 14 | 1.0 | — | 1.5 | — | — | — | — | 3.5 |

any gain during the entire period, and others gained only $\frac{1}{10}$th or $\frac{2}{10}$ths mgm. No egg gained more than $\frac{1}{10}$th mgm. in any one day. It is unlikely that the increases resulted from errors of technique or weighing, as great care was

taken to avoid these. Hence, although the gains are too small to be of much significance, they do show that the cuticle is slightly permeable to water absorption.

## Moisture Loss

The information sought in these investigations was the response of the egg to dryness as it developed from oviposition to hatching. Standard drying procedures were adopted so that direct comparisons could be made in the quantitative data obtained. Variations and differences were then checked and subjected to further experiment, with the purpose of explaining how or why they occurred. Finally, the effects of dryness on survival were studied.

Two standards of drying were used during the experiments: 25° C. and 50% R. H. for 1 to 20 days, and 40° C. and 0% R. H. for 1 hr., both in still air in Scheibler desiccators. The former exposure was moderate and was intended to dry the eggs slowly. Weighings were made initially and after 1, 2, 4, 6, 8, 10, 15, and 20 days. Comparisons of weight loss could be made at any of these exposures. A serious disadvantage lay in the fact that development usually proceeded during the exposure, so that changes in water loss associated with particular stages of development were obscured.

Eggs normally absorb water during a part of their developmental period; they absorb water at any time after the eight-day stage to recuperate from a loss or insufficient absorption; insufficient water during anatrepsis induces a diapause that in effect is a block to blastokinesis; various membranes and cells through which water transfer is accomplished are undergoing changes in permeability; all these facts complicate the situation. Though a slow dehydration such as is produced by the 25° C. and 50% R. H. exposure resembles conditions that would sometimes be found in nature, for a direct comparison a much faster desiccation is necessary. The standard used, 40° C. and 0% R. H. for one hour, was reasonably satisfactory. The temperature was not lethal and did not affect the waterproofing of the eggs; the desiccation was lethal only in eggs that had very high rates of loss.

### *Relation of Moisture Loss to Embryonic Age*

#### *Eggs of 0 to 7 Days of Age*

The only consistently uniform results were obtained with eggs under eight days of age. Table II lists the losses of such eggs at both drying conditions. All of the eggs could legitimately have been lumped, and the statement made that no loss greater than 0.3 mgm. was recorded, or that 95% or more lost 0.2 mgm. or less. However, the records are presented in more detail to show the inconsistency of the variations among ages in this comparatively homogeneous age group. Since the sensitivity of the spring torsion balance used was 0.1 mgm., variations of this amount are not significant for individuals.

It should be pointed out that the similarity in the magnitudes of losses suffered under the two drying conditions is purely coincidental. The noteworthy quantitative aspects of the data are the uniformity of the losses and their comparatively low level, each as compared with older eggs. In addition,

TABLE II

LOSSES SUSTAINED BY 0- TO 7-DAY EGGS OF *M. bivittatus* WHEN EXPOSED TO TWO ARBITRARY CONDITIONS OF DESICCATION

| Age, days | No. of eggs | Percentage losing: | | | |
|---|---|---|---|---|---|
| | | 0.0 mgm. | 0.1 mgm. | 0.2 mgm. | 0.3 mgm. |
| *At 40° C. and 0% R. H. for 1 hr.* | | | | | |
| 0 | 40 | 43 | 47 | 8 | 2 |
| 1 | 26 | 4 | 62 | 31 | 3 |
| 2 | 38 | 34 | 53 | 13 | 0 |
| 3 | 40 | 33 | 55 | 10 | 2 |
| 4 | 39 | 8 | 62 | 28 | 2 |
| 5 | 35 | 17 | 54 | 26 | 3 |
| 6 | 34 | 32 | 62 | 6 | 0 |
| 7 | 33 | 6 | 70 | 24 | 0 |
| *At 25° C. and 50% R. H. for 24 hr.* | | | | | |
| 0 | 14 | 57 | 36 | 7 | 0 |
| 1 | 41 | 10 | 44 | 44 | 2 |
| 2 | 12 | 67 | 25 | 8 | 0 |
| 3 | 15 | 40 | 53 | 7 | 0 |
| 4 | 19 | 47 | 32 | 21 | 0 |
| 5 | 15 | 40 | 40 | 20 | 0 |
| 6 | 15 | 67 | 33 | 0 | 0 |
| 7 | 20 | 20 | 40 | 35 | 5 |

however, it is unexpected to find that 6- and 7-day eggs react like younger ones, for they possess a hydropyle, a secondary lipoid layer, a yellow cuticle, and a partial white cuticle, none of which is present in younger eggs. The hydropyle is functioning, for the average 6-day egg has absorbed water to the extent of 5.3% of its original weight, and a 7-day egg 11.5%. In view of these facts one would expect greater losses of moisture, through the hydropyle, as found in eggs over seven days of age.

*Eggs of 8 to 14 Days of Age*

By the eighth day of incubation at 25° C. the effects of absorbed water are reflected in the rates of loss obtained when the eggs are subjected to desiccation. The rates increase, but they also show greater variation. Since the rates of water absorption are fairly variable among eggs of the same age, it is to be expected that losses would be similarly variable; actually they are much more so.

The influence of absorption on desiccation is readily seen in Table III, Series A, for which the rate rises suddenly at the time that the eggs are most actively absorbing water and then falls when absorption tapers off. As the ages listed are not really integers but represent variations of at least $\pm$ 0.5 days, somewhat more than half of the 8-day eggs had not yet begun to lose at

TABLE III

LOSSES SUSTAINED BY 8- TO 14-DAY EGGS WHEN EXPOSED TO 40° C. AND 0% R. H. FOR ONE HOUR

| Age, days | No. of eggs | Percentage losing: 0.0 to 0.5 mgm. | 0.6 to 1.0 mgm. | 1.1 to 1.5 mgm. | 1.6 to 2.0 mgm. | 2.1 to 2.5 mgm. | 2.6 to 3.0 mgm. | 3.1 to 3.5 mgm. | 3.6 to 4.0 mgm. |
|---|---|---|---|---|---|---|---|---|---|
| *Series A* | | | | | | | | | |
| 8 | 32 | 62 | 25 | 10 | 3 | 0 | 0 | 0 | 0 |
| 9 | 32 | 0 | 9 | 6 | 35 | 44 | 6 | 0 | 0 |
| 10 | 36 | 0 | 6 | 14 | 28 | 30 | 16 | 3 | 3 |
| 11 | 31 | 58 | 13 | 16 | 10 | 0 | 3 | 0 | 0 |
| 12 | 31 | 87 | 7 | 6 | 0 | 0 | 0 | 0 | 0 |
| 13 | 28 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 24 | 96 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Series B* | | | | | | | | | |
| 8 | 49 | 70 | 10 | 6 | 12 | 0 | 2 | 0 | 0 |
| 9 | 61 | 22 | 25 | 18 | 5 | 13 | 7 | 7 | 3 |
| 10 | 56 | 13 | 25 | 16 | 18 | 18 | 5 | 5 | 0 |
| 11 | 68 | 22 | 18 | 20 | 25 | 9 | 3 | 3 | 0 |
| 12 | 63 | 49 | 13 | 24 | 5 | 8 | 1 | 0 | 0 |
| 13 | 109 | 40 | 33 | 16 | 6 | 1 | 2 | 1 | 1 |
| 14 | 142 | 52 | 28 | 10 | 5 | 2 | 1 | 1 | 1 |

the higher rate, and by the 11th day about the same proportion had returned to the lower rate. The higher rate therefore appears to be effective for three days.

Rate changes in samples of eggs from the same pod, taken as they developed from the 8-day to the 14-day stage, were traced for several pods. In each the rate rose from the 8th to the 10th or 11th day and thereafter declined, but as usual the reactions of individual eggs were highly variable. Pods also varied in the degree of resistance to desiccation exhibited by the eggs. A representative sample is contained in Table IV, which shows the changes that took place during the aging of 30 eggs from a single pod.

Until the 10th or 11th day desiccation is fairly predictable, but beyond this time the variability is so great that it obscures the effects of many experimental treatments. The reasons for this form the basis of experimental work discussed later in this paper. The eggs of Series A, Table III, in which the desiccation rate dropped and remained low after absorption was completed, are a special case; they are tentatively considered to have reacted to desiccation in an optimum manner, though they were the only ones to do so. Series B represents all other laboratory-reared eggs and eggs collected in the field, both in the fall and in the spring. They are a random mixture, some groups of which showed tendencies to high rates of loss, some to low rates, but most

TABLE IV

PERCENTAGE WEIGHT (BASED ON AVAILABLE WATER) LOST BY 30 *M. bivittatus* EGGS FROM THE SAME POD WHEN EXPOSED TO 40° C. AND 0% R. H. FOR ONE HOUR. SAMPLES DRIED DAILY AS EGGS DEVELOPED FROM THE 8TH TO 14TH DAYS OF EMBRYONIC AGE

| Embryonic age, days | | | | | | |
|---|---|---|---|---|---|---|
| 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 14 | 38 | 66 | 56 | 36 | 23 | 26 |
| 6 | 12 | 64 | 54 | 32 | 14 | 13 |
| 4 | 12 | 44 | 31 | 26 | 13 | — |
| 3 | 12 | 30 | 28 | 10 | 13 | — |
| — | 9 | 10 | 4 | — | 11 | — |

to extreme variability. In spite of this it may be seen that the rate of loss was higher on the 9th to 11th days than afterwards.

*Eggs Over 14 Days of Age*

The large variability in desiccation rates noted above for eggs 11 to 14 days old applies equally to eggs over 14 days. Apart from the group of eggs listed as Series A in Table III, which showed consistently low desiccation rates all the way to 30 days of age, the rates were very unpredictable. Table V shows

TABLE V

LOSSES SUSTAINED BY EGGS OVER 14 DAYS OF AGE WHEN EXPOSED TO 40° C. AND 0% R. H. FOR ONE HOUR

| Age, days | No. of eggs | Percentage losing: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.0 to 0.5 mgm. | 0.6 to 1.0 mgm. | 1.1 to 1.5 mgm. | 1.6 to 2.0 mgm. | 2.1 to 2.5 mgm. | 2.6 to 3.0 mgm. | 3.1 to 3.5 mgm. | 3.6 to 4.0 mgm. |
| 15 | 56 | 61 | 21 | 5 | 2 | 4 | 4 | 0 | 3 |
| 16 | 57 | 67 | 21 | 9 | 2 | 1 | 0 | 0 | 0 |
| 17 | 55 | 47 | 18 | 20 | 13 | 2 | 0 | 0 | 0 |
| 18 | 51 | 49 | 22 | 16 | 8 | 5 | 0 | 0 | 0 |
| 19 | 37 | 35 | 30 | 19 | 5 | 3 | 5 | 3 | 0 |
| 20 | 31 | 48 | 23 | 13 | 13 | 0 | 0 | 3 | 0 |
| 21-27* | 56 | 57 | 25 | 7 | 7 | 4 | 0 | 0 | 0 |
| Diapause | 132 | 98 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

* *Prediapause and nondiapause.*

the losses of eggs over 14 days of age, not including Series A. The variability here is very great, and precludes any correlation between loss and age until diapause is imminent. After the 21st day, eggs that were to enter diapause underwent a gradual reduction of their desiccation rates, until in diapause the rate was uniformly low. The reduction appeared to correspond chronologically with the gradual onset of diapause.

## Influence of Various Factors on the Rate of Desiccation

It would be desirable to be able to interpret the drying of a grasshopper egg in terms of its age, its previous treatment, and the conditions of drying; and to go further than this and interpret these factors in terms of permeability and the forces responsible for it. This objective has not been reached, but the experiments reported herein may help toward the ultimate solution of this complex and difficult problem.

### Loss Through the Cuticle

Slifer's (9) excellent work on the function of the hydropyle has possibly led to a disregard of other means of water transfer through the egg membranes of grasshopper eggs. It has been shown herein that small amounts of water can be absorbed through the general cuticular surface. The permeability in the opposite direction is much greater, allowing considerable loss. All of the tests involving the sealing of the hydropyle were carried on with eggs of 14 days or older. Only then can the chorion be peeled off with sufficient ease to ensure that the cuticle is undamaged. The chorion must be removed before the seal is applied because it conducts moisture within its spongy interior, even under a seal applied to its outer surface. Chemical methods of removing the chorion, as in sodium hypochlorite or xylol, obviously cannot be used because of their effects on permeability; both of these chemicals increase the rate of water loss.

Tables VI and VII give some idea of the weight lost through the cuticle when the chorions were peeled off and the hydropyles were sealed with liquid solder. Fourteen-day eggs were comparatively resistant to loss through the

TABLE VI

Number of 14-day to diapause eggs losing amounts shown. Chorions of eggs peeled off, and hydropyles sealed. Exposed to 40° C. and 0% R. H. for one hour

| Age, days | No. of eggs | Number of eggs losing: | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0.0 to 0.5 mgm. | 0.6 to 1.0 mgm. | 1.1 to 1.5 mgm. | 1.6 to 2.0 mgm. | 2.1 to 2.5 mgm. | 2.6 to 3.0 mgm. | 3.1 to 3.5 mgm. |
| 14 | 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16-21 | 37 | 3 | 10 | 12 | 4 | 5 | 2 | 1 |
| Diapause | 5 | 5 | 0 | 0 | 0 | 0 | 0 | 0 |

cuticle; older eggs, however, sometimes lost large amounts when the hydropyles were sealed. The reduction in individual eggs as a result of sealing the hydropyle (Table VI) theoretically represents the loss through the hydropyle. The difference between the means is significant.

Some objection may be made to the above on the grounds that peeling the chorion off may disrupt the waterproofing layer on the surface of the cuticle. For this reason a group of 19- to 21-day eggs were tested in a different manner.

TABLE VII

LOSSES BY INDIVIDUAL EGGS BEFORE AND AFTER BEING PEELED AND SEALED. EXPOSED TO 40° C. AND 0% R. H. FOR ONE HOUR EACH TIME

| Age, days | Loss before peeling and sealing, mgm. | Loss after peeling and sealing, mgm. | Reduction, mgm. |
|---|---|---|---|
| 14 | 0.3 | 0.3 | 0.0 |
| 14 | 0.3 | 0.1 | 0.2 |
| 14 | 0.4 | 0.1 | 0.3 |
| 14 | 0.5 | 0.2 | 0.3 |
| 14 | 0.6 | 0.2 | 0.4 |
| 14 | 0.8 | 0.2 | 0.6 |
| 14 | 0.8 | 0.1 | 0.7 |
| 14 | 1.2 | 0.3 | 0.9 |
| 16 | 0.8 | 0.3 | 0.5 |
| 16 | 1.1 | 0.6 | 0.5 |
| 16 | 1.3 | 0.6 | 0.7 |
| 17 | 0.8 | 0.7 | 0.1 |
| Means | 0.74 | 0.31 | 0.43† |

† $t = 3.7^{**}$.

Thirty eggs were each covered with liquid solder over the whole surface of the chorion except posterior to the micropylar ring. This treatment left the hydropyle and the chorion above and around it unsealed. Another 30 eggs served as untreated controls. All were then exposed to 40° C. and 0% R. H. for one hour and the weight loss was recorded for each egg. As shown in Table VIII, the treated eggs lost significantly less than the controls. The differences represent the potential losses through the cuticle that were prevented by the seals.

TABLE VIII

INDIVIDUAL LOSSES BY 19- TO 21-DAY EGGS OF *M. bivittatus* AT 40° C. AND 0% R. H. FOR ONE HOUR. TREATED EGGS SEALED EXCEPT AT POSTERIOR TIPS; CHORIONS ON

| Losses, mgm. | | | | | |
|---|---|---|---|---|---|
| Treated | | | Untreated | | |
| 0.4 | 0.7 | 1.0 | 0.7 | 1.3 | 2.0 |
| 0.5 | 0.7 | 1.0 | 0.8 | 1.4 | 2.1 |
| 0.5 | 0.7 | 1.0 | 0.9 | 1.4 | 2.2 |
| 0.5 | 0.7 | 1.1 | 0.9 | 1.5 | 2.2 |
| 0.6 | 0.7 | 1.1 | 1.0 | 1.5 | 2.4 |
| 0.6 | 0.8 | 1.2 | 1.0 | 1.5 | 3.6 |
| 0.6 | 0.8 | 1.2 | 1.0 | 1.5 | 3.6 |
| 0.7 | 0.9 | 1.4 | 1.0 | 1.7 | 3.9 |
| 0.7 | 0.9 | 1.4 | 1.1 | 1.8 | 4.3 |
| 0.7 | 1.0 | 1.7 | 1.1 | 2.0 | 4.7 |
| Mean: 0.86 mgm.† | | | Mean: 1.87 mgm. | | |

† $t = 4.88^{**}$.

The same treatment was applied to 10-day eggs that were near the peak of the absorptive period and had a high desiccation rate. The mean losses at 40° C. and 0% R. H. for one hour were 3.58 mgm. for the controls and 1.01 mgm. for the treated eggs; the $t$ value for the means was 13.55**. The variability of the losses for the treated eggs was low.

Since similar results were obtained by two methods, it is concluded that water is lost in appreciable amounts through the general cuticular surface of the egg as well as its specialized area, the hydropyle.

## Effects of Removing the Chorion

It was shown in the preceding section that moisture is lost freely through the cuticle of many eggs. In some of the tests used to establish this point it was necessary to peel off the chorion. This may be done with more or less ease with turgid eggs over 13 days of age if they are surface-dried. Usually the chorion cracks open in one or more places and the peeling is readily extended from these foci. Eggs under 14 days of age are more difficult to peel and may require some drying to reduce the turgor before peeling can be successfully accomplished. Nine-day eggs cannot be peeled because the white cuticle is not yet thick enough to withstand such rough handling.

Even though an egg peels readily it may "bleed" freely. Where the forceps scrape the cuticle of a turgid egg, small droplets of liquid may immediately penetrate to the surface. These soon dry, although not as rapidly as one might expect. A droplet exuded at any one spot does not increase in size, even though it may still be wet and the egg turgid. Other droplets may be formed but the point of penetration for each seems to be sealed immediately. Eggs vary greatly in their readiness to "bleed"; some do so at the slightest touch, whereas others may be scraped vigorously without bleeding.

To determine the effect of removing the chorion, and at the same time find out whether moisture loss through the cuticle takes place through such leaks, several groups of eggs were subjected to drying tests. Peeled and intact eggs are compared in Table IX.

TABLE IX

Comparison of weight losses of peeled and intact eggs

| No. of eggs | Age of eggs | Drying conditions | "Bleeding" conditions | Mean losses, mgm. | | $t$ value |
|---|---|---|---|---|---|---|
| | | | | Peeled | Controls | |
| 28 | Diapause | 40° C. and 0% R. H. for 1 hr. | — | 0.21 | 0.15 | 1.17 |
| 20 | 17-19 days | " " " | Severe | 0.51 | 0.58 | 0.41 |
| 50 | 16-18 days | " " " | Severe | 1.04 | 0.97 | 0.41 |
| 48 | 16-18 days | 25° C. and 50% R. H. for 24 hr. | Severe | 0.74 | 0.63 | 1.15 |
| 40 | Diapause | " " " | — | 0.43 | 0.12 | 4.59** |

Only one group of eggs exhibited a significant increase in weight loss when peeled; in the other four groups peeling made no difference. In spite of severe bleeding in three of the groups, losses from peeled eggs were scarcely

greater than from the controls. There is, of course, some loss in weight from the exuded droplets, but most of this takes place before weighing; the remainder may be responsible for the slightly greater losses by some of the peeled eggs. It probably accounts for the significant differences shown by the diapause eggs dried at 25° C., which were in a low-loss category to begin with (last row, Table IX). The fact that such eggs lose no more than eggs with chorion and cuticle intact indicates that the leaks are well sealed. It may also be inferred that in most cases the chorion does not interfere with the loss of moisture. The primary wax layer and the semiwaterproof layer on the surface of the chorion do not appear to have much waterproofing value after the eggs have reached the 16-day stage, although it is possible that they retain it under certain circumstances.

One group of eggs (first row, Table IX) was dried before and after peeling so that the reactions of individual eggs could be observed. Some eggs lost more after peeling and others less; there was no correlation between first and second drying tests for the group. In this connection it is shown elsewhere in this paper that the responses of individual eggs to two similar drying tests in quick succession are far from uniform. Some eggs lose more during the first exposure, others during the second. The mean losses are statistically similar but it is obvious to the observer that unknown factors are operating to produce such variations. Though it was the object of this work to reach an understanding of such factors, this has not been found possible on account of the complexity of the problem. Basic problems of permeability that are as yet obscure in the simplest of membranes must be solved before those of compound living membranes can be understood.

## Effects of Various Chemicals

Such solvents as chloroform and xylol remove or interfere with the waterproofing of the egg membranes and result in an increased rate of drying. Newly laid eggs are especially affected, since the cuticle is not yet formed and the sole protective layer is the primary wax layer. Immersion in chloroform (at room temperature) for only 10 sec. doubled the weight loss of newly laid eggs when dried at 25° C. and 50% R. H. for 24 hr. After a one minute immersion they lost six to eight times as much as the controls.

In diapause eggs the desiccation rate was approximately doubled by immersion in chloroform or xylol for one minute. Immersion in xylol for longer periods up to 30 min. did not further increase the rate appreciably. Peeling the eggs and lightly swabbing the cuticle with chloroform or xylol increased the losses roughly in proportion to the coverage. Swabbing the hydropyle end with chloroform was twice as effective as swabbing the cephalic end, but when the chorion was present neither treatment gave results different from those for the controls.

Sodium hypochlorite also affects the transfer of water through the cuticle and the hydropyle. Its action is presumed to be the oxidation of unsaturated elements in the lipoid layer, and is proportional, within limits, to the time it

is allowed to react. In a test on diapause eggs immersed in sodium hypochlorite for four, six, and eight minutes, the weight losses at 25° C. and 50% R. H. for 24 hr. were 8, 20, and 25% respectively; untreated controls lost less than 2%. Swabbing the cuticle of peeled eggs increases the weight loss in proportion to the coverage. Immersion past the time when the last vestige of chorion disappears from the posterior pole allows the sodium hypochlorite to penetrate there. In 0- to 5-day eggs the only barrier is the vitelline membrane, which is thinner at the posterior pole than elsewhere. Disintegration of the yolk and the embryo in this vicinity may be observed shortly after the chorion has disintegrated. In eggs over six days of age the cuticular hydropyle acts as a further barrier, but penetration is not delayed for long. Immersion for over 10 min. is likely to be fatal.

### Effects of Deviation from Optimum Schedule

In searching for a reason for the highly variable desiccation rates found in older eggs it was natural to suspect that interrupted moisture supply and a resulting deviation from the optimum developmental schedule might be contributing factors. Except for Series A in Table III, in which the desiccation rate stayed uniformly low from the 13th to 30th days of incubation, all eggs over 12 days of age, whether laboratory-reared or field-collected, exhibited this variation. Although the variability is predominant it is tempting to consider the single uniform series as normal; however, with the evidence in favor of variability because of its occurrence in field-collected eggs, it is more likely that the uniform results are atypical.

Numerous drying tests were made on developing eggs that had fallen behind schedule for one reason or another. As long as they were over eight days of age and had absorbed some water, they had medium to high desiccation rates and showed the same variations within any group as did eggs reared under optimum conditions. In any case, the mere fact of being behind schedule as a result of insufficient moisture absorption or other causes cannot be blamed for the variations, since eggs reared under presumably optimum conditions also varied.

Retarded development does not affect the desiccation rates of eggs that are too young to absorb much moisture (under eight days) or older eggs that have been denied contact water. Young eggs, up to the eighth day, normally show little variation in desiccation rate. If eggs up to six days of age are removed from contact with free water and reared thereafter at a high humidity (merely to avoid excessive drying), their rate will remain low. Table X lists the weight lost at 40° C. and 0% R. H. for one hour by eggs held at 25° C. and 80% R. H. from the 6th to 14th days of incubation. Four eggs from each of six pods were dried daily.

Since the desiccation rate is usually high between the 8th and 13th days it is evident that the lack of absorption during this period is responsible, either directly or indirectly, for the continuation of the low rate. The permeability of the hydropyles did not increase automatically, even though the embryos

TABLE X

PERCENTAGE WEIGHT LOSSES (ON THE BASIS OF AVAILABLE WATER) OF EGGS INCUBATED AT 25° C. FOR FIRST SIX DAYS ON CONTACT MOISTURE AND NEXT EIGHT DAYS AT 80% R. H. SAMPLES DRIED DAILY AT 40° C. AND 0% R. H. FOR ONE HOUR

| Age, days | Days denied free water | Percentage loss |
|---|---|---|
| 6 | 0 | 3.5 |
| 7 | 1 | 2.7 |
| 8 | 2 | 2.2 |
| 9 | 3 | 2.0 |
| 10 | 4 | 2.0 |
| 11 | 5 | 2.8 |
| 13 | 7 | 3.8 |
| 14 | 8 | 3.4 |

continued to develop and the egg membranes changed greatly during the drying period. The hydropyles were potentially permeable to water uptake at any time after the eighth day, but until some water had actually been absorbed the permeability in the opposite direction was not increased.

Eggs that had reached the end of anatrepsis at a high humidity but without contact moisture were then supplied with free water and samples weighed and dried after 0, 6, 16, 24, 48, and 72 hr. (Table XI). The eggs began to absorb

TABLE XI

COMPARISON OF GAINS AND SUBSEQUENT LOSSES AS 14-DAY "DRY-REARED" EGGS ABSORBED WATER

| Time on contact water, hr. | No. of eggs | Mean gains, mgm. | Mean loss at 40° C. and 0% R. H. for 1 hr., mgm. |
|---|---|---|---|
| 0 | 48 | 0.00 | 0.08 |
| 6 | 42 | 0.07 | 0.10 |
| 16 | 38 | 0.86 | 0.19 |
| 24 | 44 | 1.18 | 0.12 |
| 48 | 43 | 2.02 | 2.21 |
| 72 | 34 | 2.32 | 2.18 |

water in about six hours and had taken up about a third of their potential increase in 16 hr. The desiccation rate, on the other hand, was not significantly changed after six hours on contact moisture and was only $\frac{1}{12}$th of its later value after 16 hr. In a similar experiment undertaken as a preliminary to the one reported but involving a smaller number of eggs, the results were even more striking. The desiccation rate had barely started to increase in 24 hr., during which time a gain of 30% was made. It is concluded, then, that the increase in desiccation rate after the 7-day stage is dependent upon and lags behind the absorption of free water by the egg.

### Effects of Absorption After Loss on Further Loss

Eggs 12 to 14 days old were dried at 40° C. and 0% R. H. for one hour, placed on contact moisture for a day, and again dried. The mean losses were 0.13 mgm. and 0.22 mgm. for the first and second dryings, respectively ($t = 2.46$*). The intervening mean gain was 0.28 mgm. Diapause eggs treated similarly showed no significant difference. It has already been shown herein that eggs denied moisture during their normal absorption period retain their low desiccation rate until free water is provided and absorption is well under way. Absorption definitely influences subsequent loss, but since age and previous moisture history are also involved no generalizations can be made.

### Comparison of Desiccation under Different Drying Conditions

The two drying conditions used in this work were, as previously explained, arbitrarily chosen. The 24-hr. exposure to 25° C. and 50% R. H. was chosen as a slow, mild desiccation and 40° C. and 0% R. H. for one hour was chosen as rapid and severe. Direct comparisons between the losses of eggs exposed to the two conditions seem to be pointless. However, when the same eggs were exposed to both drying conditions in succession some lost more under one condition and some under the other. In an experiment designed to test this, 38 diapause eggs were exposed first to 25° C. and 50% R. H. for 24 hr., then to 40° C. and 0% R. H. for one hour. Six eggs lost more during the first exposure than during the second, six lost more during the second exposure, and 26 lost the same or within 0·1 mgm. of the same weight at each exposure. In a similar experiment the 40° C. exposure was made first. Out of 16 eggs, seven lost more during the first exposure, four lost more during the second, and five lost the same amounts or nearly so. These eggs varied from 8 to 14 days of age. There is no reason to expect any similarity in the absolute losses at these two drying conditions; that it existed in these two groups of eggs was coincidental. In a third experiment, 30 16-day eggs were exposed to the 25° C. drying conditions first. This time the first losses ranged from two to six times as much as the second. In only four cases were the first losses so great that the egg had little water to lose the second time. From such results we may conclude that the permeability of an *M. bivittatus* egg is likely to change rather suddenly, in either direction, and without any apparent relation to other eggs in a supposedly similar condition.

### Effects of Continuing Dryness

Since there is a limit to the amount of moisture an egg can lose, a decreasing desiccation rate is to be expected as the exposure continues. Under many conditions, however, the rate drops before the moisture supply is appreciably depleted. Eggs of all ages that were exposed to 25° C. and 50% R. H. for 20 days usually lost more weight during the first day of desiccation than thereafter. Although there are exceptions recorded in Table XII, the data indicate a distinct reduction in moisture loss after a variable initial rate. This suggests

TABLE XII

MEAN PERCENTAGE MOISTURE LOSSES OF *M. bivittatus* EGGS EXPOSED TO 25° C. AND 50% R. H. FOR 20 DAYS; BASED ON AVAILABLE WATER REESTIMATED AFTER EACH WEIGHING

| Age, days | No. of eggs | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 to 1 | 1 to 2 | 2 to 4 | 4 to 6 | 6 to 8 | 8 to 10 | 10 to 15 | 15 to 20 |
| 0 | 11 | 2.5 | 2.6 | 1.8 | 2.9 | 2.9 | 1.0 | 0.2 | 1.2 |
| 1 | 30 | 5.5 | 4.0 | 3.5 | 3.3 | 2.4 | 1.5 | 1.0 | 0.6 |
| 2 | 12 | 2.2 | 4.1 | 4.7 | 3.1 | 0.3 | 0.6 | 1.5 | 0.6 |
| 3 | 10 | 3.1 | 1.2 | 3.0 | 1.5 | 1.1 | 0.9 | 0.8 | 1.1 |
| 4 | 19 | 2.8 | 2.3 | 1.7 | 1.9 | 1.3 | 0.7 | 0.9 | 1.5 |
| 5 | 15 | 2.9 | 2.0 | 1.4 | 0.2 | 0.0 | 0.4 | 1.9 | 0.9 |
| 6 | 15 | 1.7 | 2.0 | 1.4 | 0.2 | 0.9 | 1.6 | 0.6 | 1.3 |
| 7 | 17 | 4.8 | 2.0 | 1.7 | 0.8 | 1.3 | 0.7 | 1.6 | 0.8 |
| 8 | 14 | 4.8 | 2.8 | 2.6 | 1.9 | 2.5 | 1.5 | 2.1 | 1.3 |
| 9 | 15 | 7.8 | 2.1 | 1.8 | 2.0 | 2.0 | 2.1 | 0.6 | 1.3 |
| 10 | 23 | 14.2 | 2.3 | 2.5 | 2.4 | 2.2 | 1.7 | 1.5 | 1.5 |
| 11 | 15 | 20.5 | 3.3 | 3.4 | 3.1 | 2.2 | 1.9 | 1.9 | 2.0 |
| 12 | 20 | 10.9 | 3.4 | 3.6 | 3.4 | 4.2 | 3.8 | 3.6 | 3.3 |
| 13 | 15 | 8.3 | 4.4 | 3.5 | 3.4 | 2.7 | 3.1 | 1.8 | 2.3 |
| 14 | 15 | 15.8 | 5.2 | 4.5 | 5.3 | 3.4 | 4.2 | 3.8 | 3.5 |
| 15 | 15 | 7.7 | 4.3 | 3.7 | 4.5 | 4.0 | 3.7 | 3.7 | 2.6 |
| 16 | 15 | 5.2 | 6.0 | 4.0 | 3.4 | 3.6 | 1.6 | 2.2 | 1.1 |
| 17 | 35 | 8.1 | 6.6 | 4.9 | 4.4 | 4.2 | 3.2 | 2.1 | 1.3 |
| 18 | 15 | 10.4 | 6.2 | 4.4 | 4.4 | 4.0 | 2.3 | 2.4 | 1.6 |
| 19 | 20 | 10.5 | 4.5 | 3.9 | 3.3 | 2.9 | 1.1 | 1.3 | 1.9 |
| 20 | 20 | 11.0 | 5.9 | 4.3 | 2.4 | 2.3 | 1.1 | 1.3 | 1.5 |
| 21 | 41 | 4.7 | 3.1 | 1.5 | 1.8 | 1.6 | 1.3 | 1.6 | 1.6 |

that the dryness acts as a stimulus inducing the egg to react in such a manner as to improve its system of water conservation. After the initial decrease in rate of desiccation, there is an irregular though general further decrease. This may be attributed in part to decreasing water supply, but mainly to increasing resistance to desiccation by the egg itself, probably as a continuation of the processes that result in the greater initial drop. The importance of decreasing moisture supply is minimized because the percentages listed in Table XII were calculated on the basis of amount of moisture present at the time of previous weighing. The dry matter was estimated and moisture content recalculated after each weighing. All figures except those in the first column are therefore greater than they would be if calculated simply on the basis of initial weight or estimated initial water content.

Eggs of 0 to 6 days of age have no cuticle or hydropyle and have barely begun to absorb moisture; they survive the 20-day exposure without serious effects. Although they appear to be a relatively homogeneous group they react irregularly to desiccation. The rate declines during the exposure, but variation precludes more precise observations.

After absorption has commenced, the general level of desiccation is higher. The rate for the first day is particularly greater, increasing roughly in relation to the amount absorbed, then falling again (cf. Table III). After the first day there is considerable decrease in rate, and a further, irregular decline takes place during the rest of the exposure.

The variability seen in the table stems from several causes, one of which is experimental error. The balance sensitivity, 0.1 mgm., is large compared with the losses, often exceeding the mean daily losses. The size of the samples was not large enough to even out these errors as much as would be desirable. A second cause is the mortality and sublethal abnormalities that occurred among older eggs during the exposure. In addition, the natural variability among eggs, as to both water absorption and subsequent loss, contributed to the general irregularity.

Exposure to 25° C. and 50% R. H. slows down the development of the eggs somewhat. Young eggs usually develop to the end of anatrepsis, even without absorbing any water, provided they do not lose too much. In older eggs the amounts gained before drying and the amounts lost during it largely determine embryonic development, and often survival as well. For these reasons it is impossible to associate characteristic desiccation rates with the various embryonic stages unless the incubation conditions are continuously favorable. Since development and desiccation are both dependent on the moisture history of the egg, they cannot be independently correlated. Hence an egg reaching a certain age while being dried cannot be expected to have the same drying rate as an egg of the same age just beginning a drying exposure.

### Effects of Absorption from Salt Solutions on Subsequent Loss

The possibility that absorption from salt solutions might affect the permeability of the eggs was investigated. Eggs were reared from the 6th to 16th days, embryonic age, on filter paper moistened with solutions of sodium chloride, potassium chloride, magnesium chloride and calcium chloride (all isotonic with Ringer's solution), as well as in Ringer's, distilled water, tap water, and the mold inhibitor sodium propionate at 1 : 1000 in aqueous solution. When the eggs reached the age of 16 to 17 days, as determined by eye location, they were dried at 40° C. and 0% R. H. for one hour. The losses were characteristic of the age group and variable within each treatment; no treatment produced losses significantly different from the others. The various salts, in the concentrations used, permitted absorption of water, were not harmful, and apparently had little or no effect on permeability.

## Survival of Eggs After Desiccation

The survival of eggs of all ages from 0 to 21 days was tested by exposing them to 25° C. and 50% R. H. for 5, 10, and 20 days, and then incubating them on moist blotter. Since these were laboratory-reared eggs of known age they were in prediapause condition, and therefore hatching could not be used as a test of survival without a diapause-breaking exposure to low temperature. Such treatment was impractical because of mortality from molds and other causes. Survival was therefore calculated on the basis of observations on development, abnormalities, and heart beat during the incubation period following the drying. When eggs that had been exposed to dryness in their younger stages developed to apparently normal maturity, there was no

difficulty in labeling them as survivors. Nor was there any doubt that some eggs were dead. Between the two extremes, however, there were all degrees of survival, represented usually by corresponding degrees of abnormal development. These eggs were viable in the sense that the embryos lived for some time after the exposure, often for long periods, but they were doomed so far as hatching was concerned. For the most part the deformities or abnormalities were obvious or fairly evident, but when the desiccation was less severe it was found impossible to determine whether the egg was normal or not. Thus the quantitative values of survival are influenced by the judgment of the observer.

Five days of drying at 25° C. and 50% R. H was not particularly harmful to eggs of any age, with the possible exception of 17- and 18-day eggs, about whose condition there was some doubt. Otherwise the survival ranged from 70% to 100% for the entire series of age groups. This variation probably corresponds to that found in the desiccation rates, those eggs with high rates of loss being least likely to survive.

Ten days' drying produced little change in the results, except that most of the 17-, 18-, and 19-day eggs contained deformed embryos, though only 60% of the 20-day eggs survived. None of the 21-day eggs were killed by the treatment; these and the 20-day survivors were apparently protected by the gradual decrease in the desiccation rate accompanying the preparation for diapause. (Diapause occurs after the 21st day and its onset is gradual rather than sudden.) Eggs exposed before they were 17 days of age exhibited a variable survival, between 70% and 100%.

Twenty days' drying also left 70% to 100% survivors, but only when the eggs were exposed when 10 days old or younger, or 21 days old. Eggs exposed when 11 to 20 days of age were rendered abnormal or killed outright.

Mortality was produced in a variety of ways. In many cases, undoubtedly, death was finally attributable to lack of moisture; earlier, however, mechanical compressions and distortions usually precluded any possibility of survival. In young eggs the collapsing of the egg membranes frequently separated the yolk into two or more sections, thus starving the embryo. In older eggs, collapse resulted in flattened embryos, sometimes so thin in spots that they could readily be seen through, yet they were still alive. Such compression and distortion of tissues, organs, and appendages foretold certain death.

Eggs that were 0 to 9 days of age before drying usually completed anatrepsis and, if they had lost less than about 25% of their original weight, survived without harmful after-effects, if provided with contact water. Such eggs have absorbed no water or too little to permit blastokinesis after reaching the 14-day stage. Eggs incubated moist to 9 or 10 days of age or older have already absorbed sufficient water to allow blastokinesis to begin. However, the egg needs a good water supply to complete revolution and katatrepsis. If the supply is inadequate, development proceeds to a point and then stops. This was fatal to the eggs under the experimental conditions used, with the exception of those 9- and 10-day eggs that had lost enough water by the time

they reached the end of anatrepsis to prevent further development. In such cases a high desiccation rate is beneficial, since the egg has more chance of survival at the 14-day stage than at any subsequent stage except diapause.

Eleven- to 14-day old eggs exposed to 25° C. and 50% R. H. for 20 days all started katatrepsis and either died between the 15- and 20-day stages or became deformed. Fifteen- to 17-day eggs reached the 19- or 20-day stage before becoming deformed or being killed. Eighteen- to 20-day eggs reached the 21-day stage with the same results, except that about 40% of the 20-day eggs managed to reach diapause and survive. All eggs exposed at 21 days or later reached diapause and owed their survival to the gradually reduced desiccation rate that precedes it, and to the low rate during diapause.

The losses that could be borne before mortality ensued were also investigated. The quantitative measurements of loss were, of course, influenced by prior gains by absorption, just as in the other experimental work already discussed, but in addition the determination of mortality was often difficult and subject to doubt. The sublethal distortions and abnormalities, particularly when present in slight degree, made accurate records impossible. With highly variable weight losses and uncertain mortality figures, the relationship between them cannot be expected to be quantitatively precise. Nevertheless, when data were assembled from several different experiments there was general agreement. At any embryonic age the eggs can survive a 20% loss in weight; at no age can they withstand a loss greater than 40%. On the basis of moisture content alone, this means that an egg can lose roughly a third of its moisture without harm but cannot survive more than a two-thirds loss. Between these limits lies a zone where some eggs survive and some die, but most are rendered "potentially dead" by deformation.

The only deviation from the above conditions occurs before the eggs begin to absorb water, or during the first six or seven days. During this period the eggs survive a 15 to 20% loss in weight but are killed outright by losses over 30%. These eggs, of course, have a lower water content than postabsorption eggs, so that the limits of one-third to two-thirds, based on moisture content alone, are still applicable. During this period, also, before the cuticle is formed or adequately formed, there are no indefinite cases between survival and death. This is because the chorion is the sole covering with strength enough to retain the shape of the egg; as desiccation proceeds, the chorion of a young egg becomes indented to a limited extent and then cracks wide open, exposing the contents of the egg and of course killing it.

These general observations are further supported by data obtained from several hundred eggs of all ages exposed to 25° C. and 50% R. H. for five days. In addition, a group of field-collected eggs in postdiapause condition were all brought to the 16-day stage at the same time, weighed, and exposed to 25° C. and 50% R. H. At periods of one to eight days thereafter they were again weighed, the losses were calculated, and the eggs were then incubated on moist blotter. Since these were nondiapause eggs, hatching was used as the indicator of survival. When hatching ceased the chorions

TABLE XIII

PERCENTAGES OF HATCHED, DEFORMED, AND DEAD EGGS COMPARED WITH WEIGHT LOSSES FOR EGGS EXPOSED FOR 1 TO 8 DAYS TO 25° C. AND 50% R. H.

| No. of eggs | Weight loss, % | Hatched, % | Deformed, % | Dead, % |
|---|---|---|---|---|
| 4 | 6 – 10 | 100 | 0 | 0 |
| 16 | 11 – 15 | 94 | 6 | 0 |
| 17 | 16 – 20 | 94 | 6 | 0 |
| 15 | 21 – 25 | 73 | 20 | 7 |
| 17 | 26 – 30 | 53 | 41 | 6 |
| 10 | 31 – 35 | 50 | 40 | 10 |
| 22 | 36 – 40 | 41 | 45 | 14 |
| 19 | 41 – 45 | 11 | 68 | 21 |
| 5 | 46 – 50 | 0 | 80 | 20 |
| 8 | 51 – 55 | 0 | 50 | 50 |
| 8 | Over 55 | 0 | 0 | 100 |

were removed in sodium hypochlorite and the numbers of deformed and dead eggs counted. Most of the deformed eggs were still alive though for all practical purposes they would be considered dead. With a longer exposure or longer observation period many would have passed into the dead category. Fast-drying eggs were all included in this category; their rapid collapse prevented embryonic development and they died as 16- or 17-day embryos. Slower drying allowed development to proceed, even though abnormally, so that the eggs listed as deformed had usually completed dorsal closure (18 to 19 days).

In general, the results agree with those for survival of other ages. The rather wide range of reaction to desiccation is not surprising in view of the many factors influencing it. The drying conditions involve temperature, humidity, and time; the desiccation process is made up of numerous permeability reactions that are tied in with a few tangible things like age, development, and conditioning, but also with a host of intangible things that are associated with the permeability of all living tissues and membranes. Finally, the survival of the organism to the point of successful eclosion depends on its ability to develop normally at a reduced moisture level and to develop in spite of the mechanical distortions resulting from the collapse of the drying egg.

## Discussion

When the egg of *M. bivittatus* is laid, it is enveloped by the chorion and the vitelline membrane. Waterproofing is accomplished by the primary wax layer on the inner surface of the chorion, but it is not perfect, for small amounts of moisture are lost in unsaturated air or gained when free water is available. On the sixth day of incubation at 25° C. the serosa secretes the yellow cuticle and begins to lay down the white cuticle. The formation of the hydropyle is a part of this process and results in a permeable area in the relatively impermeable cuticle. Matthee (4), working with the eggs of the brown locust, *Locustana pardalina* (Walk.), states that the serosa secretes a secondary wax

layer that is "transferred to the microscopic space between the yellow and white layers by a system of pore canals in the white layer". This does not apply to eggs of *M. bivittatus* because the waterproofing lies on the outer cuticular surface. Swabbing the cuticle lightly with chloroform, xylol, or sodium hypochlorite resulted in an increased rate of desiccation proportional to the extent of the treatment, indicating a surface effect. Moreover, these substances do not penetrate the yellow cuticle, for they do not affect the bonding between the yellow and white layers of peeled but otherwise intact eggs. On the other hand, pieces of excised cuticle separate readily into the two layers in cold sodium hypochlorite, which can be shown to attack the bonding by penetrating the white cuticle as well as at the torn edge. In cold acetone there is no separation; in warm acetone the layers harden but can be split apart. In cold or warm chloroform the white cuticle softens and becomes sticky. It can be scraped off the yellow cuticle, but not easily. In xylol, fragments of cuticle that are still damp cannot be separated into layers. If the pieces are air-dried first, they become hard and brittle in xylol, but cannot be split.

The lipoid solvents are not satisfactory for separating the cuticular layers, but sodium hypochlorite, which is an oxidizing agent, is particularly successful. Another oxidizing agent, potassium permanganate, also results in a good separation, but whereas sodium hypochlorite seems to attack only the bonding, leaving the yellow and white cuticles apparently unchanged, potassium permanganate drastically changes both layers and perhaps the bonding as well. It is of interest to note that after treatment in potassium permanganate the white cuticle may be peeled into two or three layers. The fibrous structure is retained, however; therefore the fibers must be arranged in planes parallel to the surface.

In view of these tests, it is indicated that the bonding material separating the yellow and white cuticles is probably not lipoid, and plays no part in the cuticular waterproofing. Waterproofing is accomplished by a lipoid layer on the outside surface of the yellow cuticle, adjoining the vitelline membrane. That this is a discrete layer on the surface and not an impregnation is evident from the fact that a transition temperature zone was recorded when water loss was plotted against temperature (unpublished data). Beament (2) has shown that a lipoid layer exhibits a transition temperature but a lipoid impregnation does not.

It has been shown that the serosa becomes fully formed on the sixth day of incubation at 25° C., and that on the same day the yellow cuticle and the beginnings of the white cuticle are laid down. The cuticular lipoid layer must be secreted first, probably soon after the serosa reaches full development. The egg is thus waterproofed by the chorionic or primary lipoid layer for only five or six days, and thereafter also by the cuticular lipoid layer. The primary layer decreases in effectiveness as the egg expands (as a result of water absorption), but this is of little practical importance after the eighth day, since by then the hydropyle has taken over control of permeability.

The chorionic waterproofing is present at oviposition and the cuticular lipoid layer on or before the sixth day of incubation, the former before any absorption of water from the environment is possible, and the latter before absorption becomes appreciable. The expansion of the egg apparently ruptures the chorionic but not the cuticular waterproofing. This suggests that the cuticular lipoid layer is oily rather than waxy and that it expands with the stretching cuticle. The evidence is against progressive deposition of the lipoid, which would have to penetrate the white and yellow cuticles and their bonding material.

It is surprising that the increase in desiccation rate comes on the eighth day rather than on the sixth. One would expect the increase to coincide with the beginning of the operation of the hydropyle; if so, the hydropyle is inoperative for about two days. But this is very unlikely, for normally the egg is actively absorbing water at this time, and the experimental evidence all indicates that the hydropyle is responsible. It is nevertheless true that absorption begins before the hydropyle is formed, even though the amounts are small. It is possible that the serosa is responsible for the earliest absorption, about the fourth day, even before the serosal cells have stopped dividing. Such a possibility, however, merely widens the gap between the commencement of absorption and the increase in desiccation rate to four days, and does not help to explain the delay. Nor is the increasing turgor of the absorbing egg an explanation, for partially dehydrated eggs lose water almost as fast as turgid ones.

Many of the difficulties encountered in interpreting the quantitative results of these studies cannot be solved until more is known about the physical and chemical properties of the egg membranes. Water loss is directly controlled by these membranes, which develop, function, and in some cases degenerate as the embryo develops. Changes in desiccation rates are only indirectly related to embryonic age, but it is convenient to consider them together. Thus, eggs of *M. bivittatus* are most resistant to desiccation from the time they are laid until the embryos reach the 7-day stage. Not until they reach diapause are they again in such a secure position. Continuing dryness after oviposition is not particularly hazardous, for the embryos can usually complete anatrepsis and enter a secondary diapause. Only a severe drought would kill them at this time, for the eggs are protected by the pod and by the surrounding soil. These dry out slowly, whereas precipitation moistens them quickly, and free water is soon available to the eggs. Absorption does not begin at once, however, or at least it begins at a very slow rate, gradually increasing over a period of a day or more. This delay protects the eggs in case of a minor, temporary moistening of the soil, which would otherwise initiate the absorption and then fail, leaving the eggs with an increased desiccation rate in a drying environment.

If embryonic development has proceeded to the 8-day stage, or somewhat beyond, when dryness ensues, the fate of the egg will depend on several

factors. Desiccation will proceed at a much higher rate than in 0- to 7-day eggs, but the egg will have absorbed some water and this acts as a buffer to some extent. The more advanced the egg the more chance it has of reaching the end of anatrepsis and the protecting secondary diapause. However, if the desiccation is slight and the egg's moisture supply is still adequate when it reaches the end of anatrepsis, blastokinesis will follow. Continuing dryness under such conditions would probably be fatal.

The fate of eggs that are exposed to dryness following blastokinesis will depend on the severity of the desiccation and their moisture supply before drying. During this period the desiccation rates are so variable that conditions killing one egg may have little effect on another. The closer the egg gets to diapause or hatching, the more chance it has of survival. The desiccation rate drops gradually after the 21-day stage, and during diapause is fairly low, almost as low as for 0- to 7-day eggs.

During the period following blastokinesis, and possibly beginning before it, the general cuticular surface may become permeable to moisture loss. This additional site of potential loss adds considerably to the difficulty of interpreting the quantitative results. Attempts to explain the variability of desiccation rates were generally unsuccessful. It is not caused by retardation of development, irregular moisture absorption, intermittent drying, or combinations of these. It was probably not caused by laboratory handling techniques, since eggs freshly collected from the field reacted similarly. It could be caused by the removal of eggs from their protective pods, which is necessary for weighing, but this is unlikely. Cuticular permeability is the factor most likely to be responsible for the variability, but no indication of a relationship to known factors was found.

Recovering from the effects of desiccation by the re-absorption of water is possible and is probably common in nature; it depends entirely on the extent of desiccation. Often, as previously noted, the embryo may remain alive but be so deformed that hatching is impossible.

In conclusion, it has been shown that the reactions of grasshopper eggs to moisture are very complex. Some effects have been satisfactorily explained, but many will require a great deal of further research, particularly on membrane permeabilities.

## Acknowledgments

The early phases of this work were stimulated by the interest of Dr. R. H. Handford, Field Crop Insect Laboratory, Kamloops, B.C., then co-ordinator of grasshopper research for Western Canada, and the late H. W. Moore, who later held that position. The technical assistance of R. R. Forster, B. Fortin, A. Hewitt, and N. S. Church, Field Crop Insect Laboratory, Lethbridge, Alta., is greatly appreciated; to the last-mentioned are due special thanks for critical appraisal of the manuscript.

## References

1. BEAMENT, J. W. L. Waterproofing mechanism of an insect egg. Nature, 157 : 320. 1946.
2. BEAMENT, J. W. L. The role of wax layers in the waterproofing of insect cuticle and egg shell. Discussions Faraday Soc. 3 : 177-182. 1948.
3. JAHN, T. L. Nature and permeability of the grasshopper egg membranes: II. Chemical composition of membranes. Proc. Soc. Exptl. Biol. & Med. 33 : 159-163. 1935.
4. MATTHEE, J. J. The egg of the brown locust. Farming in South Africa, 25 (293) : 255-257. 1950.
5. SALT, R. W. Water uptake in eggs of *Melanoplus bivittatus* (Say). Can. J. Research, D, 27 : 236-242. 1949.
6. SHUTTS, J. H. An electron microscope study of the egg membranes of *Melanoplus differentialis* (Thomas). Biol. Bull. 97 : 100-107. 1949.
7. SLIFER, E. H. Insect development. IV. External morphology of grasshopper embryos of known age and with a known temperature history. J. Morphol. 53 : 1-22. 1932.
8. SLIFER, E. H. The origin and fate of the membranes surrounding the grasshopper egg, together with some experiments on the source of the hatching enzyme. Quart. J. Microscop. Sci. 79 : 493-506. 1937.
9. SLIFER, E. H. The formation and structure of a special water-absorbing area in the membranes covering the grasshopper. Quart. J. Microscop. Sci. 80 : 437-457. 1938.
10. SLIFER, E. H. The effects of xylol and other solvents on diapause in the grasshopper egg; together with a possible explanation for the action of these agents. J. Exptl. Zool. 102 : 333-356. 1946.
11. SLIFER, E. H. Isolation of a wax-like material from the shell of the grasshopper egg. Discussions Faraday Soc. 3 : 182-187. 1948.
12. SLIFER, E. H. A microscopical study of the hydropyle and hydropyle cells in the developing egg of the grasshopper, *Melanoplus differentialis*. J. Morphol. 87 : 239-274. 1950.

# USE OF DAILY METABOLIC PERIODICITIES AS A MEASURE OF THE ENERGY EXPENDED BY VOLUNTARY ACTIVITY OF MICE[1]

By J. S. Hart[2]

## Abstract

At temperatures of 10, 20, and 30° C., average metabolism of 12 mice in small respiration chambers was significantly greater during the night than during the day but was sensibly the same at night and day when constant work levels were imposed in a treadmill apparatus. In these tests the increase in metabolism due to work varied only slightly, if at all, with temperature. It is concluded that diurnal variation in metabolism in the small chambers resulted mainly from variation in voluntary muscular activity. On this supposition, it is estimated that the energy expended in voluntary activity averaged 2350 cal. per mouse per 24 hr. at all these temperatures, which was 12, 16, and 27% of the total daily expenditure at 10, 20, and 30° C. respectively.

## Introduction

The determination of voluntary activity* in absolute units presents a difficult problem. Most measurements have involved the use of mechanical devices or actograms based on direct observation of the animals. Several investigators have shown relationships between metabolism and activity for small mammals or birds (2, 5, 10, 14) and it has been shown that diurnal periodicities in metabolism are determined partly by variations in activity (3, 6, 11, 12, 15, 16). However, the writer feels that the use of daily periodicities of metabolism in animals as a means of expressing voluntary activity in absolute terms has not been fully explored.

The factors influencing daily metabolic periodicity have been summarized by Brody (7, p. 238) who stated that the heat production is the sum of the basal energy metabolism, the energy expense of muscular activity and muscle tonus, and the SDA (specific dynamic action of food). In this paper all small changes and variations of muscle tonus (4, 8, 11) are included under the term "activity"; hence that portion of the metabolism that is in excess of basal metabolism must result from energy expenditure in activity and in SDA.

Evidence has been presented to show that at least part of SDA metabolism is added to the metabolism of muscular activity (1, 9, 17, 18, 19, 20). Therefore, with activity held constant, it should be possible to determine whether the metabolic periodicity of mice fed ad libitum is associated with a significant SDA periodicity. To test this point, tests were carried out with mice performing standard grades of work during day and night at various temperatures. Estimates have also been made of the energy expended in voluntary activity at different temperatures from analysis of the metabolic periodicities.

[1] *Manuscript received September 25, 1951.*

*Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as N.R.C. No. 2628.*

[2] *Physiologist.*

* *As used here "voluntary activity" excludes activity of thermoregulation and SDA.*

## Methods

Two series of observations were made on 12 adult white mice. The first series of tests (voluntary activity) was carried out on animals placed singly in small brass respiration chambers for 24 hr. The oxygen consumption was determined over a 24 hr. period at mean temperatures of 10.3, 20.1, and 29.9° C. (average of air and wall temperatures) with 12 mice at each temperature, using a method previously described (13).

For the second series of observations (prescribed activity) the mice were tested while they were running in a rotating wheel apparatus at the same temperatures at two times of day, 10–11:30 a.m. and 11:30 pm.–1 a.m. These times were chosen to coincide as closely as possible with the trough and peak of the metabolic cycle. The test procedure was exactly as described by Hart (13) and the running speeds were 4 and 10 cm. per sec. (20 min. at each speed) with initial observations when the wheel was not in rotation.

The schedule of tests was specified by two 6 × 6 Latin squares, so that contrasts between temperatures and between night and day metabolism were balanced in respect of any consistent difference between individual mice or between groups of six mice throughout the tests. A total of 72 tests were made during voluntary activity and 72 during prescribed activity.

The mice were fed ad libitum on commercial pelleted food except when running. When not actually under test the mice were kept singly in cages on wood shavings at 20° C. with lights on from 8 a.m. to 8 p.m.

## Results

*Observations Under Conditions of Voluntary and Prescribed Activity*

The average oxygen consumption for the 12 mice engaged in voluntary activity during the 10 a.m. to 11:30 a.m. (day) and 11:30 p.m. to 1 a.m. (night) periods is shown in Table I. At all three temperatures the night

TABLE I

OXYGEN CONSUMPTION UNDER CONDITIONS OF VOLUNTARY ACTIVITY DURING THE NIGHT (11:30 P.M.–1 A.M.) AND DAY (10-11:30 A.M.), AT DIFFERENT TEMPERATURES

| Temperature, °C. | Oxygen consumption, ml. per mouse per hr. | | |
|---|---|---|---|
| | Night | Day | Difference (night–day) |
| 10.3 | 186 ± 4 | 161 ± 4 | 25 ± 6 |
| 20.1 | 132 ± 4 | 113 ± 4 | 19 ± 6 |
| 29.9 | 99 ± 4 | 71 ± 4 | 28 ± 6 |
| Average | 139 ± 2 | 115 ± 2 | 24 ± 3 |

oxygen consumption was significantly greater than the day and the difference averaged 24 ± 3 ml. per mouse per hour. The recorded difference between night and day did not differ significantly between temperatures (the maximum

discrepancy barely exceeding its standard error), suggesting the possibility of a constant day to night difference that was independent of temperature. These results confirm those previously reported (13) in which the metabolic cycle persisted at all test temperatures.

The average oxygen consumption for the mice subjected to two levels of prescribed activity and with initial observations at 0 r.p.m. is shown in Table II. These results contrast with those discussed above in that the night

TABLE II

OXYGEN CONSUMPTION AT VARIOUS RUNNING SPEEDS AND TEMPERATURES DURING THE NIGHT (11:30 P.M.–1 A.M.) AND DAY (10-11:30 A.M.)

| Running speed, cm. per sec. | Temperature, °C. | Oxygen consumption ml. per mouse per hr. | | |
|---|---|---|---|---|
| | | Night | Day | Difference (night–day) |
| 0 | 10.0 | 198 ± 7 | 191 ± 7 | 7 ± 10 |
| | 19.9 | 134 ± 7 | 125 ± 7 | 9 ± 10 |
| | 29.8 | 92 ± 7 | 75 ± 7 | 17 ± 10 |
| 4 | 10.0 | 218 ± 7 | 206 ± 7 | 12 ± 10 |
| | 19.9 | 164 ± 7 | 161 ± 7 | 3 ± 10 |
| | 29.8 | 119 ± 7 | 121 ± 7 | −2 ± 10 |
| 10 | 10.0 | 237 ± 7 | 228 ± 7 | 9 ± 10 |
| | 19.9 | 182 ± 7 | 179 ± 7 | 3 ± 10 |
| | 29.8 | 151 ± 7 | 153 ± 7 | −2 ± 10 |

minus day differences in oxygen consumption were greatly reduced or absent. When the activity wheel was not rotating (0 cm. per sec.) there was a tendency for the night oxygen consumption to be greater than that during the day. Averaged for all three temperatures the difference of 11 ml. was 1.9 times its standard error, suggestive of a real effect, but was less than half the average night minus the day difference observed in the small chambers during voluntary activity (Table I). The lowering of the night minus day difference in the wheel apparatus as compared with the small chambers is interpreted to mean that the mice did not become quiescent at any time in the former apparatus. At the constant work levels of 4 and 10 cm. per sec., the night minus day differences did not attain statistical significance either individually or collectively, and at 29.8°C. day values averaged slightly greater than night values.

From these results it is apparent that the day to night variation in the metabolic rate, which was characteristic of mice under conditions of voluntary activity, was absent in mice engaged in a constant level of muscular activity. It can be concluded therefore that the differences in day and night oxygen consumption are due largely to differences in voluntary muscular activity. Under the conditions of the experiments with ad libitum feeding, SDA does not appear to have made a significant contribution to the daily metabolic periodicity.

*The Effect of Temperature on the Oxygen Increment of Work*

The oxygen increment of work (13) is the difference in metabolism of non-working (trough of the metabolic cycle) and working mice. In Fig. 1, oxygen

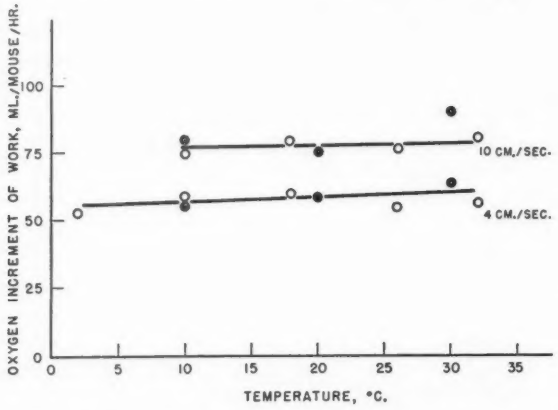



FIG. 1. Oxygen increment of work in relation to temperature and running speed. ○, Results from former study (13). ●, Present study.

increments of work are shown as the average of the day and night values at each temperature. Results obtained in a former investigation (13) are also included.

The data in Fig. 1 confirm those reported earlier (13) in suggesting that over the biokinetic range, the oxygen increment of work is little influenced by environmental temperature. At 10 and 20° C. the increases in metabolism for a given amount of work were almost identical. At 30° C. there was a suggestion of a departure in that the present series of mice running at 10 cm. per sec. showed a greater oxygen increment than did those running at the same speed at lower temperatures, the difference attaining the 5% level of statistical significance.

It has been concluded from these results that temperature has little influence on the oxygen increment of work when the level of work is low. In these mice, therefore, temperature is not a major factor modifying the daily metabolic periodicity apart from its influence on muscular activity.

*Estimation of the Energy Expended by Voluntary Activity*

Since the lowest oxygen consumption in mice usually occurs in the daytime when the animals are asleep, this level may be considered minimal for any given temperature. Any excess over the minimal level may be assumed to be derived from voluntary muscular activity. The manner of calculation, on this assumption, of excess energy expended in voluntary activity, is illustrated

in Fig. 2. The area under the metabolism curve above the minimal activity level was determined for each mouse. This excess oxygen for all the mice at each temperature was averaged (first column of Table III) at each temperature.

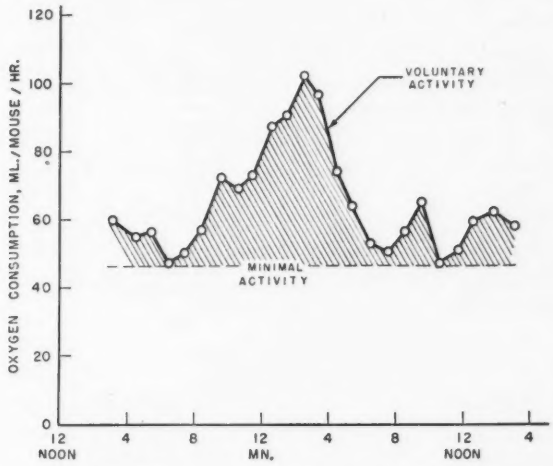



FIG. 2. Metabolic periodicity of a mouse at 30° C. Energy expended in voluntary activity over 24 hr. period is given by the shaded area under the curve.

TABLE III

METABOLISM AND ENERGY EXPENDED BY VOLUNTARY ACTIVITY OF MICE

| Temperature, °C. | Excess $O_2$ ml. per 24 hr. | Minimal $O_2$ ml. per 24 hr. | % Energy in activity | Energy per 24 hr. vol. activity, cal. |
|---|---|---|---|---|
| 10 | 487 | 4230 | 11.5 | 2370 |
| 20 | 462 | 2945 | 15.7 | 2250 |
| 30 | 502 | 1863 | 26.9 | 2440 |

These values converted to calories (fifth column of Table III) represent the energy expended in voluntary activity. In these tests the energy expended by voluntary activity at different temperatures was almost constant and averaged 2350 cal. per 24 hr. Since the minimal level of metabolism decreased with increasing temperature, the percentage of total energy expended in activity increased from 11.5 to 26.9%. A similar result was formerly described (13; Fig. 4) in which temperature had little or no effect on the day to night difference in metabolic rate.

## Summary and Conclusions

The results shown above strongly suggest that variations in voluntary muscular activity account for the observed variations in metabolism. Specific effects of temperatures and SDA appear to exert insignificant effects on metabolism of mice when fed ad libitum. Calculations have therefore been made of the energy expended in voluntary activity from the observed metabolic periodicities of the mice. The resultant figure, 2350 cal. per hr., was relatively constant at different temperatures.

These results, although providing a basis for comparative investigation to determine whether or not metabolic periodicities are quantitative reflections of activity periodicities, should not be regarded as characteristic of mice in their customary state. It was technically convenient to place the test animals in small respiration chambers, which undoubtedly restricted normal activity. Further experimentation is required with mice and other rodents free to behave without restriction in large chambers.

## Acknowledgments

The writer is indebted to Drs. R. W. Swift, J. W. Hopkins, D. Rose, F. E. J. Fry for their help with the manuscript, and to Messrs H. Pauls and G. S. Foy for technical assistance.

## References

1. ANDERSON, R. J. and LUSK, G. Animal calorimetry: the interrelation between diet and body condition and the energy production during mechanical work. J. Biol. Chem. 32 : 421-453. 1917.
2. ASHER, L. and CURTIS, G. M. Beiträge zur Physiologie der Drüsen. Untersuchungen über den respiratorischen Stoffwechsel bei Arbeit in seiner Beziehung zu den Drüsen mit innerer Secretion. Biochem. Z. 164 : 97-109. 1925.
3. BAROTT, H. G., FRITZ, J. C., PRINGLE, E. M., and TITUS, H. W. Heat production and gaseous metabolism of young male chickens. J. Nutrition, 15 : 145-167. 1938.
4. BENEDICT, F. G., LANDAUER, W. and FOX, E. L. The physiology of normal and frizzle fowl, with special reference to basal metabolism. Storrs Agr. Expt. Sta. Bull. 177. 1932.
5. BENEDICT, F. G. and RIDDLE, O. The measurement of the basal heat production of pigeons. II. Physiological technique. J. Nutrition, 1 : 497-536. 1929.
6. BLACK, A., FRENCH, C. E., COWAN, R. L., and SWIFT, R. W. Further experiments on the relation of fat to economy of food utilization. V. Fluctuations in curve of daily heat production. J. Nutrition 37 : 289-301. 1949.
7. BRODY, S. Bioenergetics and growth. Reinhold Publishing Corporation, New York. 1945.
8. BURCKARD, E., DOUTCHEFF, L., and KARPER, CH. Le rythme nycthéméreal chez le pigeon. Ann. physiol. physicochim. biol. 9 : 303-368. 1933.
9. CARPENTER, T. M. and FOX, E. L. The effect of muscular work upon the respiratory exchange of man after the ingestion of glucose and frustose. II. Heat production, efficiency, oxygen debt, excess respiratory quotient, and metabolism of carbohydrates. Arbeitsphysiol. 4 : 570-599. 1931.
10. CHEVILLARD, L. Contributions â l'étude des échanges respiratoires de la souris blanche adulte. II. Thermogénèse et thermolyse de la souris. Ann. physiol. physicochim. biol. 11 : 485-532. 1935.
11. DEIGHTON, T. and HUTCHINSON, J. C. D. Studies on the metabolism of fowls. II. The effect of activity on metabolism. J. Agr. Sci. 30 : 141-157. 1940.
12. FUHRMAN, G. J., MCLIN, E. D., and TURNER, M. L. The effect of time of day on the metabolic rate of albino mice; a manometric method. Am. J. Physiol. 147 : 284-288. 1946.

13. HART, J. S. Interrelations of daily metabolic cycle, activity, and environmental temperature of mice. Can. J. Research, D, 28 : 293-307. 1950.
14. HATFIELD, D. M. Rate of metabolism in *Microtus* and *Peromyscus*. Murrelet, 20 : 54-56. 1939.
15. HERRING, V. V. and Brody, S. Diurnal metabolic and activity rhythms. Univ. Missouri Agr. Coll. Research Bull. No. 274. 1938.
16. HORST, K., MENDEL, L. B., and BENEDICT, F. G. The effect of external factors upon the metabolism of the rat. J. Nutrition, 7 : 277-303. 1934.
17. JOHANSSON, J. E. and KORAEN, G. Wie Wird die Kohlënsaure abgabe bei Muskelarbeit von der Nährungszufuhr beeinflusst? Skand. Arch. Physiol. 13 : 251-268. 1902.
18. MEYER, F. Über die vermehrte Production spezifischdynamischer Wärme im Anschluss an muskutäre Höchstleistungen. Arbeitsphysiol. 2 : 372-386. 1929.
19. RAPPORT, D. The nature of the foodstuffs oxidized to provide energy in muscular exercise. III. The utilization of the "Waste heat" of metabolism in muscular exercise. Am. J. Physiol. 91 : 238-253. 1929.
20. WACHHOLDER, K. Der Einfluss korperlicher Arbeit auf die spezifisch-dynamische Stoffwechselsteigerung. Pflügers Arch. ges. Physiol. 251 : 485-490. 1949.

# EFFECTS OF TEMPERATURE AND WORK ON METABOLISM, BODY TEMPERATURE, AND INSULATION: RESULTS WITH MICE[1]

By J. S. Hart[2]


## Abstract

For inactive mice, metabolism below the critical temperature was proportional to the thermal gradient, and body temperature and insulation were constant in animals acclimated to 6° C., but not in those acclimated to 20° C. For active mice, the same work caused the same increase in metabolism at all temperatures, but metabolism during work was not proportional to the thermal gradient. During work, with decreasing temperature, body temperature was relatively stable in 6° C. mice but fell appreciably in 20° C. mice. Over-all insulation was lower in working than in inactive animals, but in the 6° C. acclimated mice there was a substantial improvement in insulation during work as the temperature decreased. Reports on other animals and humans also suggest that work may lower insulation. When work decreases insulation, much of the heat produced by the work, unlike that produced by SDA, may not be available for maintaining body temperature in a cold environment.


## Introduction

Increased production of heat by working and feeding may be latent at low temperatures when there is extra heat produced in maintaining body temperature (9, 10, 16). Thus excess heat resulting from activity may be utilized in temperature regulation by an animal under cold stress and in this event would be without effect on metabolism.

However, utilization of the extra heat occurs only when there is no change in heat dissipation or in body temperature and over-all insulation. Thus, extra heat produced by SDA (specific dynamic action of food) is dissipated at high temperatures but may be pooled at low temperatures with that used in thermoregulation (16). SDA therefore may not change heat dissipation and body insulation at low temperatures. On the other hand, work, because it introduces movement, may produce increased heat dissipation at all temperatures, and the heat produced may therefore not be entirely used in maintaining body temperature during cold stress.

Since data on this subject are limited, an analysis of the effect of temperature and work on metabolism, body temperature, and insulation of mice has been made. Possible effects of temperature on these physiological factors for homoiotherms in general have been also considered.

## Methods

Data on metabolic rates at different temperatures of mice maintained in single cages at 6 ± 1° C. on wood shavings have previously been reported (5) and additional results have been obtained on animals maintained at 20 ± 1° C.

[1] *Manuscript received September 25, 1951.*

*Contribution from the Division of Applied Biology, National Research Council, Ottawa. Issued as N.R.C. No. 2629.*

[2] *Physiologist.*

with the same cage conditions. The metabolic rates at different environmental temperatures (12 mice at each temperature) were taken continuously throughout a 24 hr. period when the mice were confined in metabolism chambers with food and water. Data reported are those obtained during the trough of the metabolic cycle. The condition of the mice at this period will be referred to as "inactive" and showing "minimal activity" rather than "rest" as used in previous study (5). Similarly, metabolic rates of 12 mice were determined during work, and results for a running speed of 10 cm. per sec. at each test temperature are reported.

Body temperatures at different air temperatures were obtained on additional groups of mice acclimated to 6° and 20° C. by inserting thermocouples into the colon to a depth of 2 cm. Determinations were made during quiescent periods and during running at 10 cm. per sec. The number of mice tested at each temperature ranged from 6 to 10 and body weights ranged from 23.5 to 29.5 gm.

Insulation (17) is defined as the gradient in temperature from body to air divided by the metabolic rate as derived from measurements of metabolism and body temperature (Table I). It is influenced by all factors that affect heat loss (fur, skin and tissues, posture, air movement, evaporation of water). The body surface factor generally utilized in insulation measurements is omitted here because the physiological surface area is not comparable in active and quiescent animals. Body surface is therefore treated only as one of the variables affecting insulation and heat loss.

## Results

The interrelations of metabolism, body temperature, and insulation of mice during minimal activity and work have been illustrated in Figs. 1 and 2 by plotting heat production averages and body temperature averages at different environmental temperatures. The respective ranges of variation of metabolism and body temperature data are illustrated by vertical and horizontal lines through the points.

The relation between metabolism and temperature has been simplified by assuming that it consists of two linear portions, one of which is flat and represents the lowest energy output (thermoneutral range) for any specified degree of work, and one of which is sloping and represents the influence of temperature upon metabolism. The boundary temperature of the flat and sloping portions is the critical temperature. The terms "thermoneutral range" and "critical temperature" are not used here in the restricted sense but for describing metabolism of animals during work as well as during minimal activity.

Body temperatures (Figs. 1 and 2) obtained at various air temperatures are shown along the abscissae and are joined by dotted lines to the corresponding metabolic rates. The dotted lines represent the manner in which metabolism would increase with decreasing environmental temperature if this increase

were directly proportional to the gradient in temperature from body to air (Scholander (17) ). The slopes of the lines are related to insulation and decrease with increasing insulation.

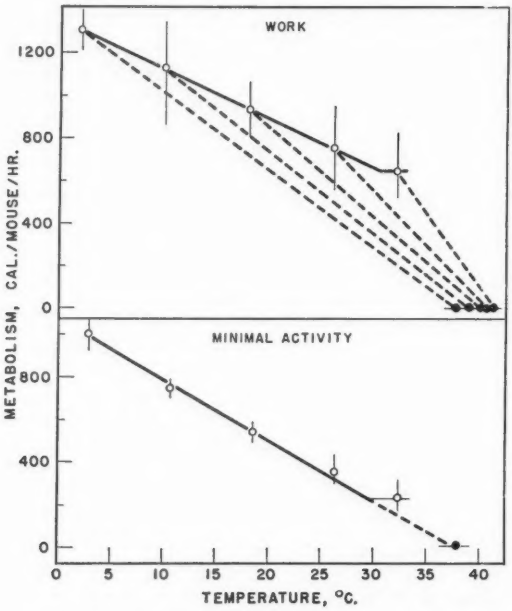



FIG. 1. Effect of temperature on metabolism (○) and body temperature (●) during minimal activity and work for mice acclimated to 6° C. Body insulation is represented by dotted lines. Vertical and horizontal bars through points are ranges of variation for metabolism and body temperature. Ranges are for single animals except for metabolism during minimal activity where they are for mice tested three at a time.

In Fig. 1 (minimal activity) it may be seen that the increase in metabolism of the 6° C. mice with decrease in environmental temperature was almost directly proportional to the thermal gradient below the assumed critical temperature. Colonic body temperatures remained constant over the whole environmental temperature range. Similar results have been reported for cold acclimated rats (4).

In Fig. 2 (minimal activity) are shown data for metabolism and body temperature of 20° C. acclimated mice. Colonic body temperatures of these mice were more variable than those of the 6° C. mice during minimal activity and there was a slight fall with lowering environmental temperature. Similarly, the increase in metabolism with decrease in environmental temperature was somewhat less than that required for proportionality with the gradient from body to air. Body insulation showed a slight increase with decreasing environmental temperature (Table I).

The effect of work on metabolism, body temperature, and insulation is illustrated in Figs. 1 and 2. Running produced an increase in metabolism at all environmental temperatures and this increase was independent of temperature (same slope of curve during work and minimal activity) in the 6° C.

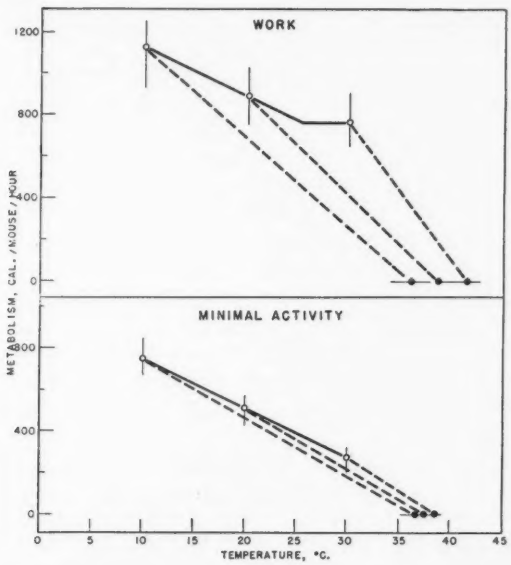



FIG. 2. Effect of temperature on metabolism (○) and body temperature (●) during minimal activity and work for mice acclimated to 20° C. Body insulation is represented by dotted lines. Vertical and horizonal bars through points are ranges of individual variation for metabolism and body temperature.

TABLE I

BODY INSULATION DURING REST AND WORK AT DIFFERENT TEMPERATURES

| Air temperature, ° C. | Insulation, ° C. per cal. per hr. | | | | | |
|---|---|---|---|---|---|---|
| | Acclimated to 6° C. | | | Acclimated to 20° C. | | |
| | Quiescent | Work | % Decrease | Quiescent | Work | % Decrease |
| 2 – 4 | 0.035 | 0.028 | 20 | — | — | — |
| 10 – 12 | 0.035 | 0.026 | 26 | 0.035 | 0.022 | 34 |
| 18 – 20 | 0.035 | 0.023 | 34 | 0.033 | 0.021 | 36 |
| 25 – 27 | 0.035 | 0.019 | 46 | — | — | — |
| 30 – 32 | 0.028 | 0.014 | 46 | 0.030 | 0.015 | 50 |

acclimated mice (5) and in the 20° C. mice with one exception. In the 20° C. mice the increase in metabolism due to work was significantly greater at 30° C. than at 10° or 20° C. Assuming a single point representation of the critical

temperature as discussed above, this is equivalent to a lowering of the critical temperature to about 26° C. Apart from these small differences in critical temperature, the metabolic rates of the 6° and 20° C. acclimated mice were almost identical. In both groups, during work, the increase in metabolism with decreasing environmental temperature was not proportional to the thermal gradient from body to air—effects which may be explained by changes in body temperature and insulation.

Body temperatures were more dependent upon environmental temperature during work than during minimal activity. At 32° C. colonic temperatures of the 6° C. acclimated mice during work (Fig. 1) averaged about $3\frac{1}{2}$° C. higher than those of inactive animals and were about 1° C. higher at 10° C. At 30° C. body temperatures of the 20° C. mice during work (Fig. 2) were also higher than those of inactive animals but at 10° C. were lower than those of inactive mice. A similar result has been obtained (6) for average body temperature. It would appear, therefore, that body temperature remained more constant during work in the 6° mice than in the 20° C. mice.

Running produced a considerable decrease in insulation (Table I) that varied from 20 to 50%. The greatest percentage decrease occurred at the higher environmental temperatures, particularly above the critical temperature. In the 20° C. acclimated mice the greatest percentage reduction in insulation produced by work occurred at 30° C. This deterioration in insulation was less at 20° C., but there was little further change at 10° C. In the 6° C. acclimated mice, the percentage decrease in insulation fell progressively with decrease in environmental temperature, until, at the lowest temperature studied, the insulation during work was as great as that for resting mice at 32° C.

The net results of changes in insulation of the two groups of mice during work were not the same. Whereas work produced approximately the same increase in metabolism at all environmental temperatures, the 6° C. acclimated mice maintained higher body temperatures than the 20° C. mice over the colder temperature range by restricting to a greater extent the deterioration of body insulation produced by work.

## Discussion

In mice, the dominant feature in the increase in metabolism produced by work was a decrease in insulation. This decrease, demonstrated or suggested also for other animals and man (1, 7, 17) results from increased air movement, increased surface area, increased evaporation, increased blood circulation, and changes in blood distribution. It therefore follows that part of the heat production of work is wasted and not available for maintenance of body temperature in cold environments.

The situation described for mice may apply for other animals that are relatively poorly insulated. Chevillard's (3) data on metabolism of resting and working mice at two air temperatures are similar to those reported herein but differ from those of Magne (14) who found that exercise at 10° C. did not

produce an increase in metabolism. Unfortunately it is not possible to assess adequately these results in the absence of information on body temperatures. Evidence obtained on pigeons (2), showing that the daily metabolic cycle persisted over a wide range of environmental temperatures, suggested that spontaneous activity produced a decrease in insulation and an increase in metabolism over the temperature range studied.

Data for ground squirrels (17) confirmed the results for mice. Oxygen consumption at environmental temperatures from 37° to − 20 °C. was measured in quiescent animals and in active animals that tried to escape. The metabolic rates of the latter were approximately double those of the former at all environmental temperatures, suggesting a decrease in insulation of about 50% if body temperatures remained constant. The critical temperature for active animals was not lower than that for quiescent animals and the metabolic rate of both quiet and active squirrels, below the critical temperature, increased with decrease in air temperature.

Work may also produce a substantial increase in heat dissipation (8) and a decrease in insulation (1) in humans at cold temperatures. Other data indicate that the rate of metabolism during work is essentially independent of environmental temperature (13, p. 420; 15), although it may increase due to shivering in seated subjects at comparable low air temperatures (11, 13, 16). These findings support the viewpoint (9, 10, 11, 12) that at low environmental temperatures the heat produced by work is utilized in maintaining body temperature. Information on this subject for humans is incomplete because these effects have not been studied over a sufficiently wide temperature range with constant clothing insulation.

*Possible Effects of Environmental Temperature on Metabolism, Body Temperature, and Insulation*

Although data for different species are limited, a consideration of certain general effects of temperature on metabolism, body temperature, and insulation under various conditions of activity appears warranted. The following diagrams (Fig. 3) illustrate some possible effects of environmental temperature on these physiological factors. In these diagrams the critical temperature is represented as a single point but the comparisons to be made also apply when there is a more gradual transition from the thermoneutral zone to the zone of thermogenesis.

In Section 1 of Fig. 3 is shown the result to be expected when there is a doubling of metabolism from the resting level BA to B′A′ while body temperature D and insulation below the critical temperature remain constant. As the environmental temperature is lowered, metabolism remains constant and insulation increases until the critical temperature is reached, after which insulation is constant and metabolism increases along BC. If metabolism is doubled to B′A′ the critical temperature will fall to B′, below which the metabolism will follow along B′C.

The above description is embodied in Rubner's "compensation theory" (16) and Lefévre's "partial substitution" hypothesis (9, 10). It may apply when

increase in metabolism is brought about by SDA, when there may be little or no change in body temperature and insulation. This metabolic picture is similar to that described by Rubner (16) for SDA of meat-feeding in dogs but there is no evidence that it applies for work.

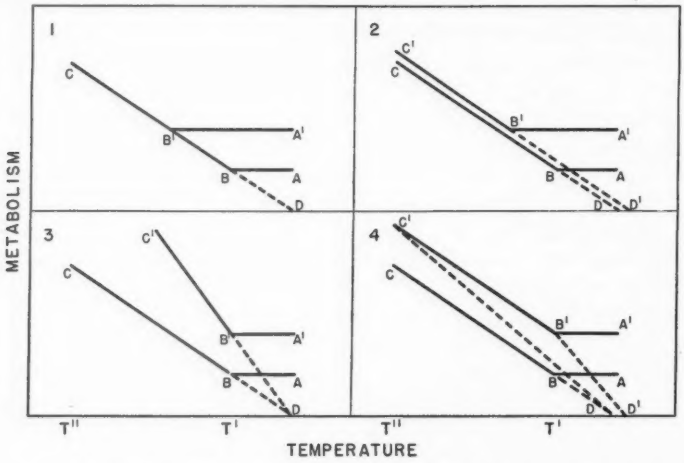



FIG. 3. Possible effects of environmental temperature on metabolism, body temperature, and insulation under various conditions of activity. Explanation in text.

In Section 2 of Fig. 3 is shown the expected result when there is an increase in metabolism and an increase in body temperature from D to D′ with no change in insulation below the critical temperature. Here again increase in heat production lowers the critical temperature from B to B′. Metabolism below B′ will proceed along B′C′ parallel to BC because there is an elevation of body temperature. The increase in metabolism along BC or B′C′ is proportional to the thermal gradient from body to air. Swift (18) has described a similar situation for rats in which the increased oxygen uptake due to feeding was about one-third as great below as above the critical temperature.

In Section 3 of Fig. 3 is shown the effect of decreasing insulation by one-half and increasing the metabolic rate with no change in body temperature. The resting metabolism is again represented by ABC and the elevated metabolism by A′B′C′. Here the line B′C′ diverges from BC with decreasing temperature, owing to decrease in insulation. During work where there is a decrease in insulation, environmental temperature might affect metabolism in the fashion shown. The effect resembles that reported by Erikson (Scholander (17) ) for ground squirrels.

The lower right hand section represents the effect of increasing metabolism when there are changes in both body temperature and insulation. The effect resembles that described for mice during minimal activity and during work. The increase in metabolism BC below the critical temperature is proportional to the thermal gradient TD only during minimal activity.

During work, metabolism above the critical temperature T′ is represented by a line B′A′ parallel to BA. In this temperature range, body temperature (D′) during work is higher than that during minimal activity (D), and variations in environmental temperature produce minimum changes in body temperature with maximum changes in insulation. During work below the critical temperature T′, increase in metabolism with decreasing environmental temperature is again represented by a line B′C′ parallel to BC. In this temperature range, changes in insulation are reduced but there may be extensive changes in body temperature. The body temperature during work (D′) is higher than that during minimal activity when the environmental temperature is elevated, but is the same (D) at a low temperature (T″). At temperature T″ the heat produced by work is entirely wasted.

These diagrams represent only a few of many possible variations in metabolism that might be produced by work at different environmental temperatures. There appears to be no theoretical reason why the increase in metabolism produced by work should be similar at all temperatures as observed for mice. Further study of other species might produce results that could be illustrated by various combinations of the lower diagrams of Fig. 3. Also, work may produce a moderate or large decrease of the critical temperature, the extent of which will depend upon its effect on body insulation.

It may be concluded that the extent to which heat produced by activity is used in the maintenance of body temperature during cold stress depends on the effect of activity on over-all body insulation. During running the utilization of heat of activity by poorly insulated animals may be minimal owing to deterioration of the insulation. Information on this subject is limited for humans and lacking for well insulated animals.

## Acknowledgments

The writer wishes to thank Dr. P. F. Scholander who inspired the writing of this paper. He is also indebted to Drs. H. J. Lips, F. E. J. Fry, and E. O. Hughes for assistance with the manuscript, and to Messrs. H. Pauls and P. D'Aoust for technical aid.

## References

1. BELDING, H. S. Protection against dry cold. Quartermaster Climatic Research Lab., Rept. No. 155, Lawrence, Mass. 1949.
2. BURCKARD, E., DONTCHEFF, L., and KAYSER, CH. Le rythme nycthéméral chez le pigeon. Ann. physiol. physicochim. biol. 9 : 303-368. 1933.
3. CHEVILLARD, L. Contributions a l'étude des échanges respiratoires de la souris blanche adulte. II. Thermogénèse et thermolyse de la souris. Ann. physiol. physicochim. biol. 11 : 485-532. 1935.
4. GELINEO, M. S. Influence du milieu thermique d'adaptation sur la thermogénèse des homéothermes. Ann. physiol. physicochim. biol. 10 : 1083-1115. 1934.
5. HART, J. S. Interrelations of daily metabolic cycle, activity, and environmental temperature of mice. Can. J. Research, D, 28 : 293-307. 1950.
6. HART, J. S. Calorimetric determination of average body temperature of small mammals and its variation with environmental conditions. Can. J. Zool. 29 : 224-233. 1951.
7. HERRINGTON, L. P. The heat regulation of small laboratory animals at various environmental temperatures. Am. J. Physiol. 129 : 123-139. 1940.

8. HORVATH, S. M., FREEDMAN, A., and GOLDEN, H. Acclimatization to extreme cold. Am. J. Physiol. 150 : 99-108. 1947.

9. LEFÉVRE, J. Chaleur animale et bioénergétique. Masson et Cie, Paris. 1911.

10. LEFÉVRE, J. and AUGUET, A. Le problème des relations entre les chaleurs du travail et du repos. Pourquoi le travail est plus économique aux basses températures. Compt. rend. 192 : 819-820. 1931.

11. LEFÉVRE, J. and AUGUET, A. La thermorégulation du travail. Rapports de ses courbes avec celles du repos. Ann. physiol. physicochim. biol. 9 : 1103-1121. 1933.

12. LEFÉVRE, J. and AUGUET, A. Les courbes thermorégulatrices et les rendements de la machine vivante dans les grandes puissances de travail. Ann. physiol. physicochim. biol. 10 : 1116-1134. 1934.

13. LUSK, G. The elements of the science of nutrition. 4th ed. W. B. Saunders Co., Philadelphia and London. 1931.

14. MAGNE, H. Influence de la température extérieure sur la grandeur de la dépense d'énergie occasionnée par le travail musculaire. Compt. rend. soc. biol. 83 : 396. 1920.

15. NIELSEN, M. Die regulation der Körpertemperatur bei Muskelarbeit. Skand. Arch. Physiol. 78 : 193-270. 1938.

16. RUBNER, M. Die Gesetze des Energieverbranches bei der Ernährung. F. Dieticke Co., Leipzig and Vienna. 1902.

17. SCHOLANDER, P. F., HOCK, R., WALTERS, V., JOHNSON, F., and IRVING, L. Heat regulation in some arctic and tropical mammals and birds. Biol. Bull. 99 : 237-258. 1950.

18. SWIFT, R. W. The effect of feed on the critical temperature of the albino rat. J. Nutrition, 28 : 359-364. 1944.

# UNIVERSITY OF CALIFORNIA

# CANADIAN JOURNAL OF ZOOLOGY

## Notes to Contributors

GENERAL: Manuscripts should be typewritten, double spaced, and the **original and one extra copy** submitted. Style, arrangement, spelling, and abbreviations should conform to the usage of this Journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts should be carefully checked before being submitted, to reduce the need for changes after the type has been set. If authors require changes to be made after the type is set, they will be charged for changes that are considered to be excessive. **All pages, whether text, figures, or tables, should be numbered.**

ABSTRACT: **An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required.**

ILLUSTRATIONS:

(i) *Line Drawings:* All lines should be of sufficient thickness to reproduce well. Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper **ruled in blue only**; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in **green, yellow, or red should not be used** unless it is desired to have all the co-ordinate lines show. Lettering and numerals should be neatly done in India ink preferably with a stencil **(do not use typewriting)** and be of such size that they will be legible and not less than one millimeter in height when reproduced in a cut three inches wide. All experimental points should be carefully drawn with instruments. Illustrations need not be more than two or three times the size of the desired reproduction, but the ratio of height to width should conform with that of the type page. **The original drawings and one set of small but clear photographic copies are to be submitted.**

(ii) *Photographs:* Prints should be made on glossy paper, with strong contrasts; they should be trimmed to remove all extraneous material so that essential features only are shown. Photographs should be submitted **in duplicate;** if they are to be reproduced in groups, one set should be so arranged and mounted on cardboard with rubber cement; the duplicate set should be unmounted.

(iii) *General:* **The author's name, title of paper, and figure number should be written in the lower left hand corner (outside the illustration proper) of the sheets on which the illustrations appear.** Captions should not be written on the illustrations, but typed together at the end of the manuscript. All figures (including each figure of the plates) should be numbered consecutively from 1 up (arabic numerals). **Each figure should be referred to in the text.** If authors desire to alter a cut, they will be charged for the new cut.

TABLES: Each table should be typed on a separate sheet. Titles should be given for all tables, which should be numbered in Roman numerals. Column heads should be brief and textual matter in tables confined to a minimum. **Each table should be referred to in the text.**

REFERENCES: **These should be listed alphabetically by authors' names, numbered in that order, and placed at the end of the paper.** The form of literature citation should be that used in this Journal. **Titles of papers and inclusive page numbers** of the references cited should be given. **All citations should be checked with the original articles.** Each citation should be referred to in the text by means of the key number.

REPRINTS: A total of 50 reprints of each paper, without covers, are supplied free to the authors. Additional reprints will be supplied according to a prescribed schedule of charges. On request, covers can be supplied at cost.

Approximate charges for reprints may be calculated from the number of printed pages, obtained by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ in. × 11 in.) and making allowance for space occupied by line drawings and halftones (not inserts). The cost per page is tabulated at the back of the reprint request form sent with the galley.

## *Contents*